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(54) Title: IMMORTALIZED HUMAN FETAL OSTEOBLASTIC CELLS

(57) Abstract

Immortalized normal human fetal osteoblastic cells that express a temperature sensitive mutant of simian virus 40 large T antigen are provided.

THYMIDINE INCORPORATION

IMMORTALIZED HUMAN FETAL OSTEOBLASTIC CELLS

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Statement of Government Rights

This invention was made with the support of the U.S. Government under National Institutes of Health Grant Nos. AR41652 and AG04875. The Government has certain rights in the invention.

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Background of the Invention

Postmenopausal osteoporosis is a condition that affects millions of women and results in debilitating bone and spinal injuries. This condition is characterized by dramatic bone deterioration due to an imbalance between

15 bone formation (by osteoblasts) and bone resorption (by osteoclasts). Osteoblasts are cells that arise from connective tissue precursor cells in bone marrow, and are associated with bone growth as they mature. Osteoclasts are cells associated with the absorption and removal of bone. In normal bone, the balance between osteoblast-mediated bone formation and osteoclast-mediated

20 bone resorption is maintained through complex cell to cell interactions. To determine the causes for bone deterioration associated with osteoporosis, and to devise effective treatments for this condition, the processes of osteoblast differentiation, osteoblast physiology, osteoblast-osteoclast communication, and osteoclast function must be studied further.

25 A number of model systems utilizing osteoblastic cells in culture have been developed to gain further insight into the process of bone formation. The most widely used cultured osteoblastic cells include primary cultures, i.e., cell cultures made by direct transfer from a natural source to an artificial medium. These cell cultures can be derived from normal human and

30 rodent bone tissue, as well as from osteosarcoma cells obtained from adult human and rodent tumors. See, for example, P.G. Robey et al., Calcif. Tissue Int., 37, 453 (1985); W.A. Peck et al., Endocrinology, 100, 1357 (1977); R.T. Franceschi et al., J. Cell Physiol., 123, 401 (1985); C.H. Heldin et al., Nature, 319, 511 (1986); S.B. Rodan et al., Cancer Res., 47, 4961 (1987); M. Kirstein

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et al., J. Cell Physiol., 134, 479 (1988); G.A. Rodan et al., in Bone and Mineral Research; W.A. Peck, Eds.; Elsevier: 244 (1984); and T.J. Martin et al., Methods Enzymol., 145, 324 (1987). These model systems, as well as other osteoblastic cell systems, have contributed greatly to the understanding

5 of osteoblast biology. However, each of these model systems has limitations with regard to its application to the study of human osteoblast biology and hormones, such as estrogen, or growth factors. Furthermore, these model systems cannot be used in methods of treating a human for bone loss.

Osteoblastic cultures derived from rodent species are

10 problematic because they may exhibit species-specific phenotypic characteristics which differ from those of human osteoblastic cultures. Osteosarcoma cells proliferate rapidly in culture but are inadequate because they have a different phenotype than untransformed cells, and an unknown genetic transforming event (G.A. Rodan et al., Crit. Rev. Eukaryot. Gene

15 Expr., 1, 85 (1991)). Thus, these cells may respond abnormally to hormone or growth factor treatment. Primary cultures derived from normal adult human bone have a normal osteoblastic phenotype but proliferate at a very slow rate and become senescent after a relatively short time in culture. Thus, as a result of the slow growth and limited life span in culture, these cells too

20 are of limited utility.

To avert the limitations of these cell systems, HOBIT (human osteoblast-like initial transfectant) cells were developed. These cells were derived from primary cultures of adult human bone cells transfected with a gene expressing the viral protein, SV40 large T antigen (P.E. Keeting et al., J.

25 Bone Min. Res., 7, 127 (1992)). Other laboratories have reported the establishment of human bone cell primary cultures infected with SV40 virus itself (H. Chiba et al., Jpn. J. Cancer Res., 84, 290 (1993)). Although these latter cells were developed from normal fetal bone cells, they are not homogeneous populations. Furthermore, while these adult and fetal SV40-

30 transformed cells express many osteoblast phenotypic markers, the expression of the SV40 large T antigen (T Ag) is constitutive, and thus cannot be manipulated. The utility of osteoblastic cells infected with wild type SV40

virus is further limited by other considerations, such as nonclonal derivation
and the expression of other viral proteins. As a result, variations in phenotype
within cell subpopulations may exist, or phenotypic changes induced by viral
infection may occur.

5   The beneficial effects of estrogen in the treatment of bone loss
in postmenopausal osteoporosis are well established. R. Lindsay,
Osteoporosis: Recent Advances in Pathogenesis and Treatment; University
Park Press: Baltimore, MD; 481 (1981); B.L. Riggs et al., J. Clin. Invest., 51,
1659-1663 (1972); and U.S. Barzel, Am. J. Med., 85, 847-850 (1988).

10   However, the mechanisms involved in the direct action of estrogen on human
bone cells remain unclear. Low levels of estrogen receptor expression have
been detected in cultured normal human osteoblastic cells as well as certain
human and rat osteosarcoma cell lines. However, because bone remodeling
does not occur in rodents, it is unclear whether estrogen effects on gene

15   expression observed in rodent model systems can be extrapolated to humans.
In addition, other species-specific differences may exist with regard to the
cellular responses to estrogen treatment. Human osteosarcoma cells
proliferate rapidly in culture, but do not display contact inhibition, nor do they
exhibit the full range phenotypic characteristics associated with normal

20   osteoblastic cells. Human osteoblastic cells are phenotypically normal and
display contact inhibition, yet proliferate very slowly and can only be
maintained in culture for a short time. The ability to study the effects of
estrogen treatment directly on human osteoblastic cells is therefore also
limited by the lack of a rapidly proliferating, yet phenotypically normal

25   osteoblastic cell model.

       Thus, what is needed are osteoblastic cells derived from normal
human cells that overcome these disadvantages. Specifically, what is needed
are phenotypically normal osteoblastic cells that could be cultured indefinitely,
proliferate rapidly, and be propagated continually using routine cell culture

30   techniques.

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Summary of the Invention

The present invention provides immortalized normal human
fetal osteoblastic (hFOB) cells. As used herein, "immortal" or "immortalized"
cells refer to a substantially continuous and permanently established cell
culture with substantially unlimited cell division potential. That is, the cells
5   can be cultured substantially indefinitely, i.e., for at least about 6 months
under rapid conditions of growth, preferably much longer under slower growth
conditions, and can be propagated rapidly and continually using routine cell
culture techniques. Alternatively stated, the cells of the present invention can
10   be cultured for at least about 100 population doublings. These cells produce a
complement of proteins characteristic of normal human osteoblastic cells and
are capable of osteoblastic differentiation. They can be used in cell culture
studies of osteoblastic cell sensitivity to various agents, such as hormones,
cytokines, and growth factors, or in tissue therapy.
15   Specifically, the present invention provides immortalized
human fetal osteoblastic cells which express a temperature sensitive *(ts)*
mutant of simian virus 40 (SV40) large T antigen (T Ag). These cells are
part of an established "cell line;" however, they are generally nontumorogenic,
i.e., they do not form tumors in mammals. They are preferably part of a
20   homogeneous population. More preferably, the homogeneous population is a
clonal population. As used herein, "clonal" cells refer to a homogeneous
population of cells derived from a single progenitor cell.
                In one embodiment of the invention, human fetal bone cells,
isolated from human fetal tissue, are transfected with a gene coding for a
25   temperature-sensitive mutant of SV40 large T antigen to yield immortalized
human fetal osteoblastic cells, designated herein as hFOB 1.19. As used
herein, "transfection" refers to a process by which foreign DNA is introduced
into eucaryotic cells and expressed. The foreign DNA is typically included in
an expression vector, such as a circular or linearized plasmid vector. In the
30   preparation of a preferred embodiment of the present invention, human fetal
bone cells are transfected by electroporation with the expression vector
pUCSVtsA58. Additionally, the human fetal tissue cells can be transfected

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with a selectable marker gene, such as a gene coding for resistance to an
agent normally toxic to the untransformed cells, such as an antibiotic, an
antineoplastic agent, or an herbicide. In the preparation of another preferred
embodiment of the present invention, human fetal bone cells transfected with

5   a gene for *ts* mutant of SV40 T Ag are also transfected with the expression
vector pSV2neo.

Although the cells of the present invention are prepared by
transfecting with the gene coding for a temperature-sensitive mutant of the
SV40 large T antigen that is incorporated into the expression vector

10   pUCSVtsA58, gene fragments or mutations of this gene can be used as long
as they impart temperature regulation to the resultant immortalized cells as
defined herein. In addition, other genes that impart temperature regulation to
the resultant cells can be used.

Although the cells of the present invention are referred to as

15   immortalized, they could alternatively be referred to as transfected or
transformed. Advantageously, they are normal cells that are conditionally
immortalized. By this it is meant that the large T antigen is capable of being
inactivated. Although the cells are still viable and express proteins, they can
be put into a state of low proliferation. That is, preferred immortalized

20   human fetal osteoblastic cells of the present invention can undergo rapid cell
division, or little or no cell division as a result of inactivating the large T
antigen. This inactivation can occur by increasing the temperature of
incubation of the cell cultures. For example, at a temperature at or less than
about 37°C, preferably about 33-36°C, rapid cell division occurs, whereas at a

25   temperature above about 37°C little or no cell division occurs. Rather,
differentiation occurs at elevated temperatures. Preferably and
advantageously, the cells of the present invention can be cycled between an
active and an inactive state, i.e., the large T antigen can be cyclicly activated
and inactivated. In this way, cells of different phenotypes can be propagated

30   from the immortalized normal human fetal cells of the present invention.

The preferred cells of the present invention have the identifying
characteristics of ATCC CRL 11372. That is, they are clonal, conditionally

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immortalized normal human fetal cells capable of osteoblastic differentiation. They differ from transformed osteosarcoma cells in that they have the ability to differentiate into mature osteoblasts expressing the normal osteoblast phenotype. Thus, the cells of the present invention provide a homogenous,

5   rapidly proliferating model system for studying normal human osteoblast differentiation, osteoblast physiology, and hormonal, growth factor, and other cytokine effects on osteoblast function and osteoblast differentiation.

The immortalized cells of the present invention express very low levels of the estrogen receptor, i.e., less than about 200 activated

10   receptors per nucleus. "Activated receptors per nucleus" is a measure of the number of estrogen receptors that are capable of binding 17β-estradiol and translocating to the cell nucleus. The number of activated receptors per nucleus is determined by the micro nuclear binding assay. D.S. Colvard et al., Clin. Chem., 34, 363-369 (1988). It is assumed that the amount of bound

15   17β-estradiol detected in this assay is equivalent to the number of functional estrogen receptors, i.e., the ligand/receptor stoichiometry is 1:1. The cells of the present invention can be transfected with a gene coding for human wild-type estrogen receptor. Transfected cells express human estrogen receptor at a level of at least about 400, preferably at least about 800, activated receptors

20   per nucleus. Significantly, these cells are responsive to estrogen treatment. The establishment of these estrogen responsive human fetal osteoblastic cells provides a model system for the study of estrogen action on osteoblast physiology, differentiation, and function, e.g., cytokine and growth factor production.

25   The estrogen responsive human fetal osteoblastic cells of the present invention can be prepared from the immortalized normal human fetal cells of the present invention and any replicable expression vector containing a gene coding for human estrogen receptor. In a preferred embodiment of this invention, this expression vector is a plasmid, preferably a plasmid having the

30   identifying characteristics of plasmid pHEGO-HYG, which is described in further detail in the examples.

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Accordingly, another aspect of the present invention provides a
method of testing a drug, i.e., a biological or chemical agent, for effects on
osteoblastic cell physiology. This method involves exposing a culture of
immortalized normal human fetal osteoblastic cells which express a

5   temperature sensitive mutant of simian virus 40 large T antigen to the drug
and monitoring at least one of the resultant changes in the physiology of the
cultured cells. These cellular changes can be any of a variety of changes of
interest. This includes, for example, monitoring secretion of growth factors
and other cytokines, growth of osteoblastic cells, expression of osteoblast

10   associated genes, formation of mineralized nodules, mineralization of an
extracellular matrix, or formation of bone. If the drug under investigation is
added to a culture of cells of the present invention at various incubation
temperatures, its effect on different osteoblastic phenotypes can be
investigated. For example, if the cells are incubated at a restrictive

15   temperature, i.e., greater than about 37°C, preferably greater than about 38°C,
and more preferably greater than about 39°C, cell division is slowed,
differentiation increases, and a more mature osteoblast phenotype is produced.
Thus, preferred embodiments of the present invention include adding a drug
for evaluation to a culture of immortalized human fetal undifferentiated

20   osteoblastic cells wherein the cells are cultured at a temperature no greater
than about 37°C. Alternatively, a drug can be added to a culture of cells
wherein the cells are cultured at a temperature greater than about 37°C for
evaluation of the drug's effect on a different phenotype, e.g., more mature
phenotype, of the cells.

25   Furthermore, the present invention is directed to a method of
treating bone loss in a mammal, e.g., a human. This method involves placing
immortalized human fetal osteoblastic cells which express a temperature
sensitive mutant of simian virus 40 large T antigen into or onto a deteriorated
bone at the point of deterioration. As used herein, "deteriorated bone" refers

30   to one that has deteriorated as a result of osteoporosis, bone fracture, bone
break, or bone loss around a surgical implant. The cells are placed into the

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bone at the point of deterioration, fracture, or break in an amount effective to
cause or induce new bone formation/replacement.

Brief Description of the Drawings

5   **Figure 1.** Thymidine incorporation of hFOB 1.19 cells cultured
at various temperatures. Subconfluent hFOB cultures were cultured at the
indicated temperatures for 24 hours, then pulsed with ^{3}H-labeled thymidine for
24 hours for each day of the time course, except that the designation 39.5/33.5
indicates that the cells were cultured at 39.5°C for 24 hours, then returned to
10   33.5°C for the remainder of the time course. Thymidine incorporation was
measured after each pulse labelling. Error bars = 1 standard deviation, n = 4.

            Figure 2. Immunostaining of postconfluent hFOB 1.19 cells for
osteoblast phenotypic markers. Postconfluent (day 8 after confluence) hFOB
15   1.19 cells cultured at 33.5°C were stained by an immunoperoxidase method
using primary antibodies (Ab) specific to: (A) osteopontin (OP); (B)
osteonectin (ON); (C) osteocalcin (OC); (D) bone sialoprotein (BSP); (E) type
I collagen; (F) no primary Ab; (G) SV40 T Ag; and (H) no primary Ab for T
Ag. Dark (i.e., red) staining indicates areas where each protein (bound by
20   Ab) is localized (100x magnification).

            Figure 3. Induction of cAMP levels in hFOB 1.19 cells by
various agonists. Confluent hFOB 1.19 cells (at 39.5°C) were pretreated with
1mM isobutylmethylxanthene (IBMX) for 2 minutes, then treated with the
25   indicated dose of each agonist for 10 minutes. Quantitation of cAMP levels
was then performed by radioimmune assay (RIA), and the amount of cAMP
(pmoles/10^5 cells) for each agonist treatment was compared to control
treatment (IBMX only) and expressed as a percentage (as denoted). Error
bars = SEM (standard error of the mean), n = number of experiments (as
30   denoted).

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Figure 4. Mineralization of the extracellular matrix by hFOB
1.19 cells. The hFOB cells were cultured at 33.5°C past confluence (day 0),
then stained by the modified von Kossa (Schenk et al., in Methods of
Calcified Tissue Preparation; G.R. Dickson et al., Eds.; Elsevier; 1-4 (1984))
5   method to visualize mineralization of the extracellular matrix on the following
days: (A) day 2; (B) day 4; (C) day 6; (D) day 8; and (E) day 10. Darkly
stained areas are nodules with mineralized matrix (25x magnification).

Figure 5. Regulation of alkaline phosphatase (AP) activity in
10   hFOB 1.19 cells. Subconfluent (A and C) or confluent (B and D) hFOB cells
were incubated at either 33.5°C (A and B) or 39.5°C (C and D) in
differentiation media, then fixed and incubated with a substrate-based stain for
AP activity in situ. Dark staining indicates high AP activity (200x
magnification). (E) Confluent hFOB cells were incubated at the indicated
15   temperatures in differentiation media (DMEM/F12 with 0.2% (v/v) charcoal-
stripped FBS (csFBS), 100 µg/ml ascorbic acid, and 10^{-8} menadione) for 24
hours, then treated for 48 hours with the indicated doses of 1,25-dihydroxy
vitamin D_3 (1,25-$(OH)_2D_3$), or ethanol vehicle (control) at the indicated
temperatures. AP activity in cell extracts was then measured using the p-
20   nitrophenol phosphate assay and normalized to total protein. Units = µmoles
p-nitrophenol/hour at 37°C. Error bars = 1 standard deviation, n = 6
experiments. ** = P < .01 vs control, $ = P < .001 39.5°C vs 33.5°C, two-
tailed student's paired T test.

25   Figure 6. Regulation of osteocalcin (OC) expression in hFOB
1.19 cells. Confluent hFOB 1.19 cells were incubated at the indicated
temperatures in differentiation media (DMEM/F12 with 0.2% (v/v) charcoal-
stripped FBS, 100 µg/ml ascorbic acid, and 10^{-8} menadione) for 24 hours, then
treated for 48 hours with the indicated doses of 1,25-dihydroxy vitamin D_3
30   (1,25-$(OH)_2D_3$), or ethanol vehicle (control) at the indicated temperatures. OC
levels in hFOB-conditioned media were measured by RIA using a ^{125}I-labelled
antibody to human OC and normalized to total protein in cell extracts. Error

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bars = 1 standard deviation, n = 6 experiments. * = P < .05 39.5°C vs 33.5°C,
** = P < .01 39.5°C vs 33.5°C, two-tailed student's paired T test.

Figure 7. Construction of the pHEGO-HYG vector. The
5   human estrogen receptor (hER) cDNA was inserted upstream of the CMV
promoter and downstream of the SV40 polyadenylation signal. The direction
of transcription for the ER and hygromycin resistance (HYG^r) genes are
indicated with arrows. Selected restriction enzyme sites are denoted (Cla =
*Cla*I, Sac = *Sac*I, BamH = *Bam*HI, Bcl = *Bcl*I, Kas = *Kas*I, Nru = *Nru*I), as
10 well as the map location (in Kilobase pairs from the *Cla*I site).

Figure 8. Northern analysis of the hFOB/ER subclones. Total
RNA (10 µg) isolated from the indicated hFOB/ER subclones was fractionated
by glyoxal-agarose electrophoresis, blotted to a nylon filter, and hybridized
15 with the ER and glyceraldehyde 3-dehydrogenase (GAPDH) cDNA probes.
The ER mRNA steady state levels were determined by densitometry of the
autoradiogram, normalized to GAPDH levels, and expressed as a ratio
(ER/GAPDH) of relative mRNA levels (REL. RNA LEVEL). * - Indicates a
truncated size ER mRNA.
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Figure 9. Analysis of 17β-estradiol (E$_2$) effects on
progesterone receptor levels in hFOB/ER9 cells. The amount of specific
progesterone binding was measured in control (CNT) or E$_2$-treated hFOB/ER9
cells by the micro nuclear binding assay (panel A) or the dextran coated
25 charcoal assay (panel B) as described in the examples section. Control and
E$_2$ treatments were ethanol vehicle and 10^{-9}M E$_2$, respectively, for 96 hours.
Each bar denotes the mean value from three separate assays +/- SEM.

Figure 10. Northern analysis of E$_2$ effects on *c-fos* mRNA
30 levels. Total RNA (10 µg) was isolated from control (CNT) and E$_2$-treated
hFOB/ER cells cultured at the indicated temperatures (33.5°C and 39.5°C).
Control cells were treated with ethanol vehicle for 30 minutes, whereas E$_2$

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treatments were for 30 or 60 minutes as denoted. Following gel
electrophoresis and northern blotting, the RNA was hybridized to *c-fos* and
ribosomal protein S14 (rpS14) cDNA probes. The relative mRNA level
(REL. RNA LEVEL) as measured by densitometry was expressed as a ratio
5   (FOS/S14).

Detailed Description of the Invention

The present invention is directed to human normal fetal
osteoblastic cells that are conditionally immortalized with a gene coding for a
10   temperature-sensitive (ts) mutant (*ts*A58) of SV40 large T antigen (T Ag).
This gene product, termed early gene or large T antigen, is responsible for
immortalizing cells; however, the mechanism by which this occurs is
unknown. The expression vector alone has no effect. Expression of T Ag in
human cells results in an increased and prolonged rate of proliferation, which
15   is believed to result from interaction of T Ag with the retinoblastoma gene
product, Rb. Under conditional immortalization, however, the mutant T Ag is
only active (i.e., the cells are immortalized) at a temperature referred to herein
as "permissive" temperature. Thus, the return to an inactive
(nonimmortalized) state can be manipulated by changing the incubation of the
20   cells to a restrictive (nonpermissive) temperature. This type of conditional
immortalization has been used successfully in other cell types. See, for
example, J.Y. Chou, Mol. Endocrinol., 3, 1511 (1989).

Thymidine incorporation experiments indicate that expression of
this gene at the permissive temperature, which is at or less than about 37°C,
25   preferably about 33-36°C, and more preferably at a temperature of about
33.5°C, results in rapid cell proliferation. When hFOB cells are cultured at an
elevated temperature, i.e., at a temperature greater than about 37°C, preferably
at about 38°C, cell proliferation slows considerably. Typically, proliferation
does not occur at a restrictive temperature of 39.5°C. These results suggest
30   that the conditional immortalization is dependant on a functional large T
antigen. The resumption of rapid cell proliferation following conversion from

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the restrictive to the permissive temperatures is consistent with this
hypothesis.

The differentiation of osteoblastic cells in culture involves a
programmed developmental sequence. This sequence is characterized by an

5   early proliferative stage during which osteoblastic cells are relatively
undifferentiated, and later postconfluent stages which involve the expression
of bone cell phenotypic markers and ultimately extracellular matrix
mineralization. See, for example, M.A. Aronow et al., J. Cell Physiol., 143,
213 (1989); and G.S. Stein et al., FASEB J., 4, 3111 (1990).

10   There are numerous phenotypic markers and characteristics
associated with osteoblast differentiation. These include: the expression of
alkaline phosphatase (AP), osteopontin (OP), osteonectin (ON), osteocalcin
(OC), bone sialoprotein (BSP), and type I collagen; an increase in cellular
cAMP (cyclic adenosine menophosphate) levels in response to parathyroid

15   hormone (PTH); an increase in OC levels in response to 1,25-(OH)$_2$D$_3$
treatment; and the formation of a mineralized matrix. hFOB cells express
high levels of all of the above-mentioned osteoblast-associated proteins in
postconfluent cultures. The expression of these phenotypic markers is
indicative of stages of osteoblast differentiation which follow confluency in

20   culture. Further, OC levels increase in response to 1,25-(OH)$_2$D$_3$ treatment,
and cAMP levels increase in response to PTH treatment in postconfluent
hFOB cells. These data suggest that hFOB cells contain functional vitamin D
receptors and PTH receptors. In addition, hFOB cells form mineralized
nodules in postconfluent cultures, which is characteristic of the late stages of

25   osteoblast differentiation in culture. Therefore, hFOB cells appear to be
relatively undifferentiated cells programmed to differentiate upon reaching
confluence into cells which possess the full spectrum of osteoblast-associated
features.

The conditional nature under which the hFOB cells of the

30   present invention are immortalized prompted further investigation into the
effect of cell proliferation on the expression of genes associated with
osteoblast differentiation. Both alkaline phosphatase and 1,25-(OH)$_2$D$_3$-

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induced osteocalcin expression in postconfluent hFOB cells are significantly
affected by incubation temperature. Since the incubation of hFOB cells at a
restrictive temperature of about 39.5°C results in the inactivation of the
temperature sensitive SV40 T Ag and a subsequent decrease in cell

5   proliferation, the effect on AP and OC gene expression suggests that changes
in T antigen activity and subsequent effects on cell proliferation affect cell
differentiation. See Table 1 for a summary of hFOB characteristics. It is
possible that AP and OC expression are regulated by nuclear factors such as
FOS and JUN, which are associated with cell proliferation. However, it is

10   also possible that retinoblastoma may directly affect OC and AP gene
expression, since the inactivation of SV40 large T Ag would affect Rb
activity. However, the invention is not limited by any particular mechanistic
theory of action.

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TABLE 1

Effects of Temperature and Cell Density on hFOB Cell Functions			
A.	Temperature	33°C	39°C
	Proliferation	++	-
	AP	+	++
	1,25 D_3 action on OC gene	+	+++
	1,25 D_3 action on AP gene	+	+
	E_2 action on c-fos gene	-	++
	TGF-β action on c-fos, Jun B	-	+
B.	Cell Density	Subconfluent	Confluent
	Proliferation	++	±
	AP	+	++
	Mineralization/nodule formation	-	++
	Matrix production	-	++

As stated above, the cells of the present invention can be used
in cell culture studies of osteoblastic cell sensitivity to various agents, such as

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hormones, cytokines, and growth factors, or in tissue therapy. For example,
they can be used in testing drugs by adding a drug to a culture of cells of the
present invention at various incubation temperatures. In this way the drug's
effect on different osteoblastic phenotypes can be investigated.

5   Specifically, the cells of the present invention can be stably
transfected with, among other medically important genes, the gene for human
wild-type estrogen receptor (hER), utilizing the cloning techniques described
below. This makes them especially advantageous for use in medical research
and treatment. Since untransfected hFOB 1.19 cells express very low levels

10   of ER (less than about 200 activated receptors per nucleus), this invention is
also directed toward the construction of subclones of hFOB 1.19 which are
stably transfected with an ER expression vector. These estrogen-responsive
human fetal osteoblastic subclones are referred to herein as hFOB/ER
subclones. hFOB/ER stable transfectants express estrogen receptor at a level

15   greater than about 400 activated receptors per nucleus, and preferably greater
than about 800 activated receptors per nucleus. It is possible that these cells
express greater than about 10,000 activated receptors per nucleus. They are
responsive to treatment with 17β-estradiol. 17β-estradiol (E_2) is a clinically
important estrogen, and the term estrogen-responsive means responsive to

20   treatment with E_2. Generally, when the term estrogen is used, it refers to
17β-estradiol, although other estrogens may elicit similar responses. These
responses, which are expected in estrogen target cells, include increases in
endogenous progesterone receptor levels and *c-fos* steady state mRNA levels
following E_2 treatment of hFOB/ER cells. The stably transfected hFOB/ER

25   cells are extremely useful for studies of estrogen effects on osteoblast gene
expression and physiology, and hence in the diagnosis and treatment of
osteoporosis. For example, hFOB/ER cells that express moderate levels of the
estrogen receptor (e.g., about 400 to about 3,000 activated receptors per
nucleus) can be advantageously used as a model system to study the

30   physiological effects of various drugs on osteoblast function since cultured
human bone cells are known to express about 1600 ± 400 activated receptor
molecules per nucleus (E.F. Ericksen et al., Science, 241, 84-85 (1988)).

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Cells that express higher levels of ER (e.g., greater than about 3000) can also
be advantageously used to investigate osteoblast function and gene response,
and are more conveniently amenable to molecular biology experiments.

The cells of the present invention can also be used in a method

5   of treating bone loss, e.g., resulting from osteoporosis, a fracture or break,
and/or bone loss around the site of a surgical implant. This method involves
placing immortalized human fetal osteoblastic cells which express a
temperature sensitive mutant of simian virus 40 large T antigen into or onto a
deteriorated bone at the point of deterioration. Specific methods and

10   techniques used in such a tissue therapy regime are generally described by R.
Langer et al., Science, 260, 920 (1993), which is incorporated herein by
reference.

*Cloning, transfection, transformation and expression of vector
DNA in immortalized hFOB cells.* The conditionally immortalized cells of

15   the present invention are constructed using standard molecular biology
techniques that include the use of replicable expression vectors (described
below) to express genes of interest in the host cell. Construction of suitable
vectors employs standard ligation techniques known in the art. Isolated
plasmids or DNA fragments are cleaved, tailored, and relegated in the form

20   desired to generate the plasmids required.

Plasmids are transfected into host cells, preferably human fetal
bone cells or the resultant conditionally immortalized hFOB cells of the
present invention. Transfection refers to the taking up of an expression vector
by a host cell. Many methods of transfection are known to one of ordinary

25   skill in the art, e.g., calcium phosphate treatment, nuclear injection, protoplast
fusion, or electroporation. Preferably, electroporation is used. Successful
transfection is generally recognized when any indication of the operation of
this vector occurs within the host cell. Successful transformation occurs when
the DNA so introduced into the cell is replicable, either as an

30   extrachromosomal element or by chromosomal integrant. Host cells are
transfected and preferably transformed with the expression vectors of this
invention and cultured in conventional nutrient media modified as appropriate

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for inducing promoters, selecting transformants, or expressing the genes encoding the desired sequences.

The human host cells used to produce the gene product of interest may be cultured in a variety of commercially available media.

5   Preferably Delbecco's modified Eagles medium Ham F12 (DMEM/F12) (Sigma Chemical Co., St. Louis, MO) is used. In addition, any suitable custom-prepared media such as that described in, e.g., Ham et al., Methods Enzymol., 58, 44 (1979) or Barnes et al., Anal. Biochem., 102, 255 (1980), may be used as culture media for the host cells. Any of these media may be

10   supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium, calcium, or magnesium chloride or phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually

15   present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be

20   apparent to the ordinarily skilled artisan.

Plasmids from the transformants are isolated, analyzed by restriction endonuclease digestion, and/or sequenced by methods known in the art. See, e.g., J. Messing et al., Nucl. Acids Res., 9, 309 (1981) and Maxam et al., Methods Enzymol., 65, 499 (1980).

25   *Construction of replicable expression vectors.* Nucleic acid (e.g., cDNA or genomic DNA) comprising the gene of interest is inserted into a replicable vector, preferably a circular or linearized plasmid vector, for expression of the gene product. Preferably, DNA encoding a gene product that confers immortality, more preferably conditional immortality, on the host

30   cell is inserted into the expression vector. Most preferably, this DNA encodes a temperance sensitive SV40 large T antigen. In addition thereto, other expression vectors containing DNA encoding proteins, e.g., growth factors and

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hormones, preferably the human estrogen receptor, can be constructed and used to alter the cell phenotype. Further, additional expression vectors containing genes conferring antibiotic resistance, such as neomycin resistance, or other marker genes may preferably be used in the same host cell.

5   Many expression vectors are available, and selection of the appropriate vector depends on the size of the nucleic acid to be inserted into the vector and the host cell to be transformed with the vector. Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organism but can be transfected into another organism for

10   expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

Replicable expression vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin

15   of replication, one or more marker genes, an enhancer element, a promoter and a transcription termination sequence. In mammalian cell expression, a native signal sequence may be satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders,

20   for example, the herpes simplex gD signal. Generally, the origin of replication component is not needed for mammalian expression vectors, although the SV40 origin is typically used because it contains the early promoter (see below).

The selection or marker gene encodes a protein necessary for

25   the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not

30   available from complex media. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug

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resistance and thus survive the selection regimen. Examples of such dominant

selection use the drugs neomycin, mycophenolic acid, or preferably,

hygromycin. These three examples employ bacterial genes under eukaryotic

control to convey resistance to the appropriate drug G418 or neomycin

5   (geneticin), xgpt (mycophenolic acid,) or hygromycin, respectively.

               Expression vectors usually contain a promoter that is

recognized by the host organism and is operably linked to the gene of interest.

Promoters are untranslated sequences located upstream (5') to the start codon

of a structural gene (generally within about 100 to 1000 bp) that control the

10   transcription and translation of a particular nucleic acid sequence to which

they are operably linked. Such promoters typically fall into two classes,

inducible and constitutive. Inducible promoters are promoters that initiate

increased levels of transcription from DNA under their control in response to

some change in culture conditions, e.g., the presence or absence of a nutrient

15   or a change in temperature. At this time a large number of promoters

recognized by a variety of potential host cells are well known in the art.

These promoters are operably linked to the gene of interest by removing the

promoter from the source DNA by restriction enzyme digestion and inserting

the isolated promoter sequence into the vector. Both the native promoter

20   sequence and many heterologous promoters may be used to direct

amplification and/or expression of the gene of interest. Heterologous

promoters are preferred, as they generally permit greater transcription and

higher yields of expressed protein as compared to the native promoter.

Numerous promoter sequences are known for eukaryotes. Virtually all

25   eukaryotic genes have an AT-rich region located approximately 25 to 30 bases

upstream from the site where transcription is initiated Another sequence

found 70 to 80 bases upstream from the start of transcription of many genes is

the CXCAAT region where X may be any nucleotide. At the 3' end of most

eukaryotic genes is an AATAAA sequence that may be a signal for addition

30   of the poly A tail to the 3' end of the coding sequence. Any of these

sequences is suitably inserted into eukaryotic expression vectors.

Transcription from vectors in mammalian host cells is controlled, for example,

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by promoters obtained from the genomes of viruses such as polyoma virus,
fowlpox virus, adenovirus (such as Adenovirus 2) bovine papilloma virus,
avian sarcoma virus, Simian Virus 40 (SV40), hepatitis-B virus and,
preferably, cytomegalovirus; from heterologous mammalian promoters, e.g.,

5   the actin promoter or an immunoglobulin promoter; from heat-shock
promoters; and from the promoter normally associated with the gene of
interest, provided such promoters are compatible with the host cell systems.
The early and late promoters of the SV40 are conveniently obtained as an
SV40 restriction fragment that also contains the SV40 viral origin of

10   replication. Fiers et al., Nature, 273, 113 (1978); Mulligan et al., Science,
209, 1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78, 7398-
7402 (1981). The immediate early promoter of the human cytomegalovirus is
conveniently obtained as a HindIII E restriction fragment. Greenaway et al.,
Gene, 18, 355-360 (1982).

15   Transcription of the gene of interest by higher eukaryotes is
often increased by inserting an enhancer sequence into the vector. Enhancers
are cis-acting elements of DNA, usually about 10-300 bases long, that act on
a promoter to increase its transcription. Enhancers are relatively orientation-
and position-independent, having been found 5' to the transcription unit,

20   within an intron as well as within the coding sequence itself. Many enhancer
sequences are now known from mammalian genes (globin, elastase, albumin,
alpha-fetoprotein, and insulin). Typically, however, an enhancer from a
eukaryotic cell virus is used. Examples include the SV40 enhancer on the late
side of the replication origin (bp 100-270), the cytomegalovirus early

25   promoter enhancer, the polyoma enhancer on the late side of the replication
origin, and adenovirus enhancers. The enhancer may be spliced into the
vector at a position 5' or 3' to the gene being transfected, but is preferably
located at a site 5' of the promoter.

                 Expression vectors used in eukaryotic host cells will also
30   contain sequences necessary for the termination of transcription and for
stabilizing the transcribed mRNA. Such sequences are commonly available
from the 5' and, occasionally, 3' untranslated regions of eukaryotic or viral

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DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of mRNA. Preferably, the SV40 polyadenylation signal is used.

5   The invention has been described with reference to various specific and preferred embodiments and will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modifications on the basic theme of the present invention beyond that shown in the examples and detailed description, which are within the spirit and scope of the present invention.

10

Experimental Examples

Example I.
Construction and Characterization of hFOB 1.19 Cells

15   **A. Materials Used**

Delbecco's modified Eagles medium Ham F12 (DMEM/F12 1:1 w/w) mix, menadione (vitamin K_3), ascorbic acid (vitamin C), human parathyroid hormone fragment 1-34 (PTH 1-34), prostaglandin type E_2 (PGE$_2$ 11,15-dihydroxy-9-oxoprosta-5,13-dien-1-oic acid), forskolin (7β-acetoxy-

20   1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), 10x trypsin-EDTA and the alkaline phosphatase enzyme assay kit were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal Bovine Serum (FBS) was purchased from Flow Laboratories Inc. (McLean, VA) and 1,25-dihydroxy vitamin D_3 (1,25-(OH)$_2$D$_3$) was purchased from Biomol (Plymouth Meeting, PA).

25   Neomycin G418 (geneticin) was purchased from Gibco Laboratories (Gaithersburg, MD) and electroporation cuvettes were purchased from Bio-Rad Laboratories (Hercules, CA). The substrate-based staining kit for alkaline phosphatase was purchased from Vector Laboratories Inc. (Burlingham, CA) and the human osteocalcin RIA kit was purchased from Immutopics (San

30   Clemente, CA). The cAMP assay kit was purchased from Amersham Corp. (Arlington Heights, IL) and the peroxidase immunostaining kit was purchased from Biomeda Corp. (Foster City, CA). The SV40 T Ag-specific monoclonal

antibody was purchased from Oncogene Science (Uniondale, NY) and ^{3}H-labelled thymidine was purchased from New England Nuclear Research Products, DuPont Company (Boston, MA). Polyclonal antibodies specific for human osteopontin (OP) LF-7, osteonectin (ON) LF-BON, osteocalcin (OC)

5   LF-32, bone sialoprotein (BSP) LF-100, and type I collagen LF-67 were obtained from Dr. Larry W. Fisher at the Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892. See also, L.W. Fisher et al., J. Biol. Chem., 262, 9702 (1987), which is incorporated herein by reference. The T Ag expression vector

10   pUCSVtsA58 is available from D. Wynford-Thomas without restriction of use (Department of Pathology, CRC Thyroid Tumour Biology Research Group, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, United Kingdom). See also D. Wynford-Thomas et al., Mol. Cell. Biol., 10, 5365 (1990); P. Tegtmeyer, J. Virology, 15, 613 (1975); and A.J. Ridley et

15   al., EMBO J., 7, 1635 (1988). The neomycin resistance expression vector pSV2neo was purchased from American Type Culture Collection (Rockville, MD).

B. Methods Used

20   ## 1. Isolation, Transfection and Screening of hFOB Cells

Limb tissue was obtained from a spontaneous miscarriage under institutionally approved protocols as a source for primary cultures. The limb tissue was placed in phosphate buffered saline and homogenized with scalpel blades, then digested with collagenase (0.5 mg/ml) for 30 minutes and trypsin

25   (10 mg/ml) for 30 minutes. The cells were then seeded into 100 mm tissue culture plates containing phenol red-free DMEM/F12 media with 10% (v/v) FBS and 10% (v/v) human serum. The cells were incubated at 37°C in 5% CO_2 humidified air and the media was replaced every 48 hours until sufficient numbers of cells were present for the transfection procedure. Cells which did

30   not adhere to the culture plates were discarded. Adherent cells were removed from the culture plates with trypsin-EDTA treatment. Briefly, cells were rinsed with phosphate buffered saline (PBS). Then a 10x stock solution of

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trypsin-EDTA was diluted 10 fold with PBS and enough 1x solution was added to cover the surface of the culture plate and incubated at 37°C until the adherent cells were released. The cells were then rinsed with serum-containing media and centrifuged (5 minutes at 900 x g), then rinsed again

5   with serum-free media and centrifuged (5 minutes at 900 x g).

The pellet of cells (containing approximately 8 x 10^6) was resuspended in 0.4 ml of serum-free media containing 10 µg linearized T Ag expression vector pUCSVtsA58 and 2 µg linearized neomycin resistance expression vector pSV2neo (P.J. Southern et al., J. Mol. Appl. Gen., 1, 327

10   (1982)). The cell/DNA suspension was placed in an electroporation cuvette and incubated at 4°C for 10 minutes. The cells were then subjected to a pulse of 900 V/cm at 960 µFD using a Biorad electroporation device and incubated at 4°C for 10 minutes more following the pulse. The cells were then seeded into tissue culture plates containing DMEM/F12 media with 10% (v/v) FBS at

15   37°C. After 48 hours and every 48 hours thereafter, the media was replaced with fresh media containing 600 µg/ml neomycin G418. After 7-10 days of neomycin selection, resistant colonies were visible and were maintained in media containing 300 µg/ml neomycin G418. More than 50 neomycin resistant colonies were passaged by trypsin-EDTA treatment in glass cylinders

20   and seeded into separate tissue culture wells for further growth and screening.

When sufficient numbers of cells from each colony were obtained, they were screened for alkaline phosphatase activity by a substrate-based staining technique, as recommended in the substrate-based staining kit for alkaline phosphatase purchased from Vector Laboratories Inc. Briefly,

25   confluent cells were rinsed twice with PBS and fixed with absolute ethanol. Then alkaline buffer (100 mM Tris-HCl pH 9.5) containing substrate was added to the cells and incubated at 37°C for 30 minutes. Five of the colonies which were screened had high levels of AP activity (were heavily stained). The highest activity was observed in the clone hFOB 1.19.

2. Thymidine Incorporation

The hFOB cells were seeded at low density (5 x 10³ cells/well, 48 well dish) in the DMEM/F12 media with 10% (v/v) FBS and cultured for 16-18 hours at 33.5°C. The cells were then cultured for 24 hours at the test

5 temperature (33.5°C, 38.0°C, 39.5°C), and then pulsed with 0.5 μCi of ³H-labelled thymidine for 24 hours for each day of the time course. After each thymidine pulse, the cells were rinsed three times with 10% (w/v) trichloroacetic acid, then solubilized in 0.2% (w/v) sodium hydroxide. The solute was then mixed with scintillation cocktail for quantitation of ³H in a

10 Beckman model LS2800 scintillation counter.

3. Karyotype Analysis

Karyotype analysis was performed in the Mayo Cytogenetics Laboratory by the method described in J.L. Spurbeck et al., Cancer Genet.

15 Cytogenet., 32, 59 (1988), as detailed for fibroblast cultures, which is incorporated herein by reference. Briefly, hFOB cells were seeded at low density on glass coverslips in tissue culture dishes. At 10-30% confluence, the cells were treated with 0.25 μg/ml colcemid for 30-60 minutes. The cells were then prepared for metaphase spreads using a Tecan model 505 robotic

20 sample processor.

4. Immunocytochemistry

Immunostaining for SV40 T Ag was performed by fixing subconfluent hFOB cells with 100% methanol for 10 minutes at 4°C, then

25 blocking in 1% bovine serum albumin (BSA) in PBS for 60 minutes. Primary antibody (in 1% BSA in PBS) was added and incubated on the cells at 25°C for 60 minutes. The cells were rinsed with PBS and a secondary antibody (goat anti-mouse IgG) was added to the cells and incubated for 30 minutes at 25°C. Then peroxidase reagent and chromogen reagent were added according

30 to the manufacturers' specifications. Immunostaining for OP, OC, ON, BSP, and type I collagen were performed in the same manner except that day 8

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(postconfluent) cells were fixed in absolute ethanol, blocking was done with 10% (v/v) FBS in PBS, and the secondary antibody was goat anti-rabbit IgG.

5. cAMP Quantitation

5   The hFOB cells were cultured in 6 well dishes to confluence (approximately 5 x 10⁵ cells/well) at 33.5°C in DMEM/F12 media with 10% (v/v) FBS, then the culture media was replaced and the cells were incubated at 39.5°C for 48 hours. The cells were pretreated with 1 mM isobutylmethylxanthene (IMBX) for 2 minutes. The cells were then treated

10   with either 1-34 PTH (1-100 nM), PGE_2 or forskolin (10 μM) for 10 minutes. The media were removed from the cells and the cells were rinsed immediately with cold (4°C) PBS, scraped from the tissue culture dish in cold (-20°C) 70% (v/v) ethanol, transferred to 1.5 ml microcentrifuge tubes, and sonicated on ice. The cell lysate was lyophilized in a speed vac concentrator and

15   redissolved in 0.25 ml of Tris-EDTA buffer (50 mM Tris, pH 7.5, 4 mM EDTA). A portion (20-50 μl) of each lysate was mixed with 50 μl ^{3}H-cAMP and 100 μl cAMP-binding protein solution and incubated at 4°C for 120 minutes. The binding reaction was then mixed with 100 μl activated dextran-coated charcoal and centrifuged at 12,000 x g for 5 minutes. A portion (200

20   μl) of the supernatant was then mixed with scintillation cocktail for quantitation of ^{3}H.

6. Staining of Mineralized Matrix

Postconfluent hFOB cells were fixed in 1% (w/v)

25   paraformaldehyde in Tris buffered saline (TBS = 20 mM Tris, pH 7.4, 0.15 M NaCl) and rinsed with TBS. The cells were then stained by the von Kossa procedure as modified by R.K. Schenk et al. in Methods of Calcified Tissue Preparation; G.R. Dickson, Eds.; Elsevier; 1-4 (1984), which is incorporated herein by reference. Briefly, the cells were treated with 5% (w/v) silver

30   nitrate in the dark for 15 minutes. The cells were then rinsed with distilled water, subjected to ultraviolet light for 5 minutes, treated with sodium

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carbonate/formaldehyde solution for 2 minutes, and finally treated with
Farmer's reducer as described for 30 seconds.

7. **Alkaline Phosphatase and Osteocalcin Assays**

5   The hFOB cells were cultured in 6 well dishes to confluence in
DMEM/F12 media with 10% (v/v) FBS at 33.5°C, then rinsed with about 3
ml serum-free media twice. The media was replaced with differentiation
media (DMEM/F12 with 0.2% (v/v) charcoal-stripped FBS (csFBS), 100
μg/ml ascorbic acid, and 10^{-8} menadione) and the cells were incubated at the
10   desired temperature for 24 hours. The media was replaced again with
differentiation media and the cells were treated with various doses of 1,25-
$(OH)_2D_3$ or ethanol vehicle for 48 hours. The media was removed and
utilized for osteocalcin assays while the AP reaction was initiated by rinsing
the cells twice with PBS and adding 0.5 ml of alkaline lysis buffer (0.75 M 2-
15   Amino-2-methyl-1-propanol, pH 10.3) containing p-nitrophenol phosphate
substrate (2 mg/ml), and incubating at 37°C for 30 minutes. The reaction
solution was mixed with an equal volume of 50 mM NaOH, then diluted 1:40
with 20 mM NaOH. The absorbance at 410 nm was determined and
compared to p-nitrophenol standards. A portion of the reaction solution was
20   used to determine total protein concentration by the Bradford method, as
disclosed by M.M. Bradford, Anal. Biochem., 72, 248 (1976), which is
incorporated herein by reference.

        A portion of conditioned media (400 μl) or media plus known
OC standards (provided in kit) were mixed with a ^{125}I-labelled antibody to
25   human osteocalcin (anti-OC) and a plastic bead coated with the anti-OC
antibody. These mixtures were incubated 18-24 hours at 25°C, then the beads
were rinsed with wash buffer as specified by the manufacturer in the
osteocalcin RIA kit purchased from Immutopics. Briefly, the binding reaction
buffer was removed and washed three times with 2 ml each time of a wash
30   buffer. The rinsed beads were put into scintillation cocktail for quantitation of
[125]I. The control and treated samples were compared to the standard curve for
quantitation of OC.

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C. Results

1. Isolation, Transfection and Screening of hFOB Cells

Primary cultures isolated from fetal tissue were transfected with a gene coding for a temperature sensitive mutant (tsA58) of SV40 large T antigen, along with a gene coding for neomycin (G418) resistance. Individual neomycin resistant colonies were screened for alkaline phosphatase (AP) specific staining. The clone with the highest AP level, hFOB 1.19, was examined further for other osteoblast phenotypic markers. Thymidine incorporation experiments indicated that incubation of hFOB 1.19 cells at a permissive temperature of 33.5°C resulted in rapid cell division, whereas little or no cell division occurred at a restrictive temperature of 39.5°C. Measurement of AP activity in hFOB cell extracts indicated that cells cultured at 39.5°C had 2-3 fold higher levels of AP than cells cultured at 33.5°C. Further, AP activity increased in a dose-dependant manner following treatment with 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) when cultured at either temperature. Similarly, radioimmunoassay (RIA) analyses showed that the level of osteocalcin secreted from 1,25-$(OH)_2D_3$-treated hFOB 1.19 cells was 10 fold higher when the cells were cultured at 39.5°C compared to cells cultured at 33.5°C. In addition, osteocalcin levels in hFOB 1.19-conditioned media increased in a dose-dependant manner following 1,25-$(OH)_2D_3$ treatment at both 39.5°C and 33.5°C incubations. Treatment of hFOB 1.19 cells with 1-34 parathyroid hormone (PTH) at 39.5°C resulted in a 3 fold increase in cAMP levels as measured by RIA. Upon reaching confluence, hFOB 1.19 cultures went through programmed differentiation and formed mineralized nodules as observed by von Kossa staining. Further, immunostaining of postconfluent, differentiated hFOB cells showed that high levels of osteocalcin, osteopontin, osteonectin, bone sialoprotein, and type I collagen were expressed.

2. Temperature Control of hFOB Cell Proliferation

Since hFOB 1.19 cells were transfected with a temperature sensitive mutant tsA58 of SV40 large T antigen, the effect of incubation

temperature on hFOB 1.19 cell proliferation was examined. The rate of thymidine incorporation by hFOB 1.19 cells cultured at various temperatures was measured to indirectly reflect the rate of hFOB 1.19 cell division. These data (Figure 1) indicate hFOB cells cultured at the permissive temperature of

5   33.5°C proliferated rapidly with a doubling time of about 36 hours. Similarly, microscopic inspection indicated an increase in cell number from less than about 20% confluence to greater than about 80% confluence during the time course. In contrast, hFOB 1.19 cells cultured at the restrictive temperature of 39.5°C did not appear to proliferate during the four day time course, whereas

10 cells cultured at 38.0°C proliferated very slowly, with a doubling time of greater than 96 hours. However, when the cells were switched back to 33.5°C after 24 hours at 39.5°C, proliferation resumed and attained a rate similar to that observed with cells grown continuously at 33.5°C.

15 **3.   Karyotype Analysis**

In order to characterize the chromosomal makeup of the hFOB 1.19 cells, karyotype analysis was performed on passage 12 cells. The data from 100 metaphases indicate that 43% of the cells were diploid and 57% were tetraploid. Among a group of 12 diploid metaphases analyzed further, 7

20 were normal 46,XX and 5 were 44-46,XX with an 18q+ translocation. Chromosome polymorphism patterns were consistent with a clonal cell population. The hFOB 1.19 clone has been cultured at 33.5°C up to passage 30 (approximately 100 population doublings) without crisis. At passage 32 to 34 the cells entered crisis and proliferation slowed considerably.

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4.   Expression of Osteoblast Phenotypic Markers and SV40 T Antigen

To determine whether hFOB 1.19 cells express proteins which are characteristic of the osteoblast phenotype and to confirm that the transfected SV40 large T antigen (T Ag) gene is expressed, immunostaining

30 was performed with antibodies specific to osteopontin (OP), osteonectin (ON), osteocalcin (OC), bone sialoprotein (BSP), type I collagen, and T Ag. The results of these immunostaining experiments (Figure 2) showed that high

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levels of the differentiation markers OP, ON, OC, BSP, and type I collagen were expressed in postconfluent hFOB cells cultured at 33.5°C. In addition, high levels of T Ag were localized in the nuclei of hFOB cells.

5.   5.   Induction of cAMP Levels by PTH and PGE$_2$

Since cAMP levels have been shown previously to be affected by PTH and PGE$_2$ in other osteoblastic cell lines, these cAMP agonists were examined for their activity in hFOB 1.19 cells by radioimmunoassay. The data (Figure 3) indicate that cAMP levels increased more than 3 fold when

10   hFOB 1.19 cells were treated with 10 nM or 100 nM PTH, but increased less than 2 fold when treated with 1 nM PTH. Dramatic increases in cAMP levels were observed following treatment with 100 nM PGE$_2$ (> 50 fold increase) or with the known agonist 10 μM forskolin (> 80 fold increase).

15   6.   Formation of Mineralized Nodules

Many osteoblastic cell lines have been shown to form mineralized nodules during the processes of cell differentiation and matrix mineralization. Upon culturing hFOB 1.19 cells beyond confluency (day 0) at 33.5°C, formation of mineralized nodules occurred gradually until nodules

20   were clearly visible by day 8-10. Nodule formation was also observed in postconfluent cells cultured at 39.5°C. To visualize calcium deposition within the nodules, the cells were stained by the von Kossa procedure and examined under light microscopy. These data (Figure 4) showed that calcium deposition within the nodules was easily detectable by day 4-6 and became extensive by

25   day 8-10 post confluency. Interestingly, nodule formation and calcium deposition were extensive even without the addition of β-glycerophosphate or high doses of glucocorticoids to the culture media.

7.   Regulation of Alkaline Phosphatase Activity

30   Since initial screenings of neomycin-resistant transfectants indicated that the highest level of AP was present in hFOB 1.19 cells, the regulation of AP in this clone was examined by performing substrate-based

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staining and standard enzyme assays under various culturing conditions. To determine whether AP activity within hFOB 1.19 cells changes upon reaching confluency or changes by incubation temperature, substrate-based staining was performed. These data (Figure 5, panels A-D) indicated that AP activity

5   increased dramatically upon reaching confluency at either incubation temperature (33.5°C or 39.5°C). This reflects a change in the differentiation state of hFOB 1.19 cells to a more mature phenotype. The amount of AP-specific staining was clearly much higher in confluent cells (Figure 5, panels B and D) than subconfluent cells (Figure 5, panels A and C). Although there

10   appeared to be a slightly higher level of AP activity in hFOB 1.19 cells cultured at 39.5°C (Figure 5, panels C and D) compared to cells cultured at 33.5°C (Figure 5, panels A and B), this difference may be at the limit of sensitivity for this assay. In order to quantitate changes in AP activity resulting from modification of the incubation temperature or by the addition

15   of 1,25-(OH)$_2$D$_3$ to the media, standard enzyme assays with hFOB 1.19 cell extracts were performed. These data (Figure 5, panel E) showed that AP activity was 2-3 fold higher in hFOB 1.19 cells cultured at 39.5°C compared to cells cultured at 33.5°C. In addition, treatment of hFOB 1.19 cells with 1,25-(OH)$_2$D$_3$ resulted in a dose-dependant increase in AP activity in cells

20   cultured at either temperature (33.5°C or 39.5°C). This effect of 1,25-(OH)$_2$D$_3$, was relatively modest as the highest dose (100 nM) produced less than a 2 fold increase in AP activity.

8.   **Regulation of Osteocalcin Expression**

25   To determine whether hFOB 1.19 cells secrete the osteoblast specific protein osteocalcin (OC) and to determine whether OC production is regulated by 1,25-(OH)$_2$D$_3$ or by incubation temperature, radioimmunoassays with hFOB 1.19-conditioned media were performed. These data (Figure 6) indicated that OC levels in hFOB 1.19-conditioned media was near or below

30   the levels of detection without 1,25-(OH)$_2$D$_3$ treatment when the cells are cultured at either temperature (33.5°C or 39.5°C). However, treatment of hFOB 1.19 cells with 1,25-(OH)$_2$D$_3$ resulted in a dramatic increase in OC

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levels in a dose-dependant manner. Treatment with doses of $1,25\text{-}(OH)_2D_3$ as low as 0.1 nM resulted in a notable increase in OC levels, particularly when the cells were cultured at 39.5°C. Similarly, treatment with higher doses of $1,25\text{-}(OH)_2D_3$ resulted in correspondingly higher levels of OC. Interestingly,

5   the effect of $1,25\text{-}(OH)_2D_3$ on OC levels was more pronounced when the hFOB 1.19 cells were cultured at 39.5°C. In fact, when the cells were treated with 100 nM $1,25\text{-}(OH)_2D_3$, the level of OC secreted by cells cultured at 39.5°C was 10 fold higher than cells cultured at 33.5°C.

10   **D. Deposit of Cells**

     The immortalized human fetal cells hFOB 1.19 were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 USA on June 4, 1993, and assigned accession number ATCC CRL 11372.

15

Example II.

**Construction and Characterization of
Estrogen-Responsive Immortalized Human Osteoblastic Cells**

20   **A. Materials Used**

     The phenol-guanidine isothiocyanate (TRI reagent) solution for RNA isolation was purchased from Molecular Research Center (Cincinnati, OH), radiolabeled nucleotides and steroids such as $[\alpha\text{-}^{32}P]\text{-dCTP}$ (deoxycytidine triphosphate), $[^{3}H]\text{-}17\beta\text{-estradiol}$; and $[^{3}H]\text{-R5020}$

25   (progesterone receptor agonist) were purchased from Dupont-NEN (Boston, MA). The cloning vector pBluescript SK and Quickhyb buffer were purchased from Stratagene (La Jolla, CA), and Geneticin from Gibco-BRL (Gaithersburg, MD). Tissue culture media, 10X trypsin-EDTA reagent, and unlabeled $17\beta\text{-estradiol}$ were purchased from Sigma Chemical (St. Louis,

30   MO). Hygromycin B and fetal bovine serum was purchased from Flow-ICN (Costa Mesa, CA). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

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B. Methods Used

1. Vector Construction

Vector construction was accomplished utilizing standard molecular biology procedures known to one skilled in the art. See, e.g.,

5   _Current Protocols in Molecular Biology_; F.M. Ausubel et al., Eds.; John Wiley & Sons: New York, NY; 1989. Briefly, the cDNA sequence coding for the wild type human estrogen receptor (ER) was excised using _Eco_RI from the HEGO vector (L. Tora et al., _EMBO J._, 8, 1981-1986 (1989)). This 1.9 Kb fragment was ligated into the _Eco_RI site of the pBluescript SK vector and

10   excised once again using _Eco_RV and _Bam_HI. This fragment was ligated into the _Eco_RV and _Bam_HI sites of the expression vector p636, a derivative of pHYG (B. Sudgen et al., _Mol. Cell. Biol._, 5, 410-413 (1985)) containing the CMV promoter (D.R. Thomsen et al., _Proc. Natl. Acad. Sci. USA_, 81, 659-663 (1984)) inserted into the _Cla_I/_Hin_dIII sites, the SV40 polyadenylation

15   signal (_Bam_HI/_Bcl_I fragment) inserted into the _Bam_HI site, and the hygromycin B resistance gene driven by the thymidine kinase promoter. The ER expression vector resulting from the insertion of the ER cDNA into the p636 vector was designated pHEGO-HYG (see Figure 7 for plasmid map).

20 ### 2. Stable Transfection

Clonal hFOB 1.19 (Example I) cells were transfected with the ER expression vector pHEGO-HYG (ATCC deposit #79994) by electroporation as described in Example I. Briefly, 10 µg of pHEGO-HYG vector (linearized with _Nru_I) was added to the transfection mixture prior to

25   electroporation. Transfected cells were plated in growth media (Delbecco's modified Eagles medium (DMEM)/Ham F12 medium (F12) 1:1 v/v supplemented with 10% v/v charcoal stripped fetal bovine serum (csFBS)) and incubated at 33.5°C for 48 hours, then incubated in selective media containing 150 µg/ml hygromycin B for 7-10 days until resistant colonies were clearly

30   visible. Resistant colonies (~200-400 cells) were trypsinized in glass cloning cylinders as described in Example I. They were passaged and maintained in selective media containing 100 µg/ml hygromycin B until sufficient numbers

32

of cells (~2 x 10^7) were obtained for cryopreservation. Routine growth conditions included media changes every 2 days and changes to selective media containing 300 μg/ml Geneticin (G418 neomycin) instead of hygromycin B were performed every alternate media change to maintain

5   neomycin resistance.

3. Steroid Binding Assays

Specific 17β-estradiol (E_2) and progesterone (Pg) binding in hFOB/ER cells (i.e., cells that have been transfected with pHEGO-HYG) was

10   measured using the micro nuclear binding (NB) assay (D.S. Colvard et al., Clin. Chem., 34, 363-369 (1988)) and the dextran-coated charcoal (DCC) assay (S.N. Thibobodeau, et al., Clin. Chem., 27, 687-691 (1981)). Briefly, hFOB/ER cells were grown to confluence in DMEM/F12 + 10% (v/v) csFBS. The cells were then rinsed three times with serum free media (DMEM/F12 +

15   0.25% (w/v) BSA), treated with 10^-9 M 17β-estradiol or ethanol vehicle in serum free media for 48 hours, and treated again in fresh serum free media for another 48 hours. Following the 96 hour treatment period, the cells were rinsed three times with phosphate buffered saline (PBS), removed from the tissue culture flasks by trypsin/EDTA treatment, rinsed with 10 volumes of

20   DMEM/F12 + 10% (v/v) csFBS medium at 4°C, and centrifuged at 900 x g for 10 minutes at 4°C. The cell pellet was rinsed with 10 ml of PBS and centrifuged at 900 x g for 10 minutes at 4°C again. The resultant cell pellets containing 4-8 x 10^6 cells for the nuclear binding assay or 40-60 x 10^6 cells for the DCC assay were incubated with radiolabeled steroids as described

25   previously (D.S. Colvard et al., Clin. Chem., 34, 363-369 (1988); S.N. Thibobodeau et al., Clin. Chem., 27, 687-691 (1981)).

4. Northern Analyses

Total RNA was isolated from hFOB/ER cells by

30   phenol/guanidine isothiocyanate method of P.A. Chomczynski (Biotechniques, 15, 532-536 (1993)), except that an additional extraction with one volume of chloroform was added after the phenol extraction. Purified RNA samples

33

were denatured in glyoxal/dimethyl sulfoxide buffer and separated by glyoxal-
agarose gel electrophoresis (G.K. McMaster et al., Proc. Natl. Acad. Sci.
USA, 74, 4835-4838 (1977)). The RNA was then blotted to nylon filters by
capillary diffusion in 20X SSC (3M NaCL, 0.3M sodium citrate ph 7.0), and

5   bound to nylon by vacuum baking at 80°C for two hours. Hybridization was
performed in a hybridization incubator at 65°C for two hours in 10 ml
Quickhyb buffer containing 100 μg/ml denatured calf thymus DNA. Each
hybridization contained approximately 10^7 cpm (5-10 ng) of ^{32}P-labeled
cDNA. Labeling of cDNAs was performed with a random primer labeling kit

10   (Dupont-NEN, Boston, MA) in accordance with the manufacturer's
instructions using [^{32}P]-dCTP (3000 Ci/mmol), and the labeled cDNAs were
then purified by gel filtration chromatography. The northern blots were
exposed to Kodak X-OMAT AR5 film with intensifying screens at -70°C then
developed in a Kodak X-OMAT M20 film processor. Quantitation of band

15   intensities were performed with a Shimadzu (Kyoto, Japan) CS 9000 flying
spot scanning laser densitometer.

C. Results

1.   **Expression of Estrogen Receptor (ER) and E₂ Binding Activity**
20   **in hFOB/ER Subclones**

Following stable transfection of the ER expression vector
pHEGO-HYG (Figure 7) into hFOB 1.19 cells, the hFOB/ER subclones were
screened for expression of ER mRNA by northern analysis. Five subclones of
hFOB/ER cells were found to express significant amounts of the expected 1.9

25   Kb ER mRNA (Figure 8). A broad range of ER steady state mRNA levels
were expressed in the hFOB/ER subclones. The highest level of ER mRNA
expression was in hFOB/ER9, which was ~5 fold more than the level of ER
mRNA expression in hFOB/ER3, which had the lowest level. The hFOB/ER6
subclone expressed an aberrant size mRNA, so this subclone was not

30   examined further.

To determine if the hFOB/ER cells exhibited functional estrogen
binding, nuclear binding assays were performed with each subclone. These

34

data (Table 2) showed that the number of estrogen binding sites, i.e., activated

estrogen receptors, in each subclone was proportional to the amount of ER

mRNA expressed in each subclone, with the highest number of E_2 binding

sites, i.e., estrogen receptors, (9,799) in hFOB/ER9, and the lowest number

5   (825) in hFOB/ER3. Untransfected cells exhibited less than 200 activated

receptors per nucleus. Since the hFOB/ER9 cells contained the highest

number of E_2 binding sites, this subclone was examined further for estrogen

responsiveness. DCC assays performed using hFOB/ER9 cells measured ER

at a level of 312 (±29 SEM from three separate assays) fmol/mg cystolic

10   protein in this subclone. Base level ER expression in untransfected cells is

not determinable using the DCC assay because it is below the sensitivity limit.

DCC assays were not performed on subclones ER2, ER3, and ER4.

TABLE 2

Subclone	Untransfected Cells	ER2	ER3	ER4	ER9
Relative RNA level[a]	N.A.	4.0	1.0	2.9	4.7
Relative estrogen binding[b]	N.A.	3.7	1.0	3.7	11.9
Activated ERs per nucleus[c]	<200	3,076 ±280	825 ±18	3,082 ±967	9,799 ±860

[a] Arbitrary densitometry units from northern blots using ER/GAPDH ratio.
[b] Arbitrary units corresponding to relative amount of [^{3}H]-17β-estradiol molecules/nucleus.
[c] Number of bound 17β-estradiol molecules in nucleus; 1:1 stoichiometry for E_2:ER. Values are ± standard deviation (n = 4 determinations).

30

2.   **Effect of E_2 on Progesterone Receptor (PR) Levels in**
       Subclone hFOB/ER9

       E_2 treatment of normal human osteoblast-like (hOB) cells is

known to result in increased levels of endogenous PR expression (E.F.

35   Erickson et al., Science, 241, 84-86 (1988)). To determine if endogenous PR

expression is upregulated in E_2-treated hFOB/ER cells, nuclear binding and DCC assays were performed using the hFOB/ER9 subclone. These data (Figure 9) indicated that the number of progesterone binding sites localized to the nucleus increased ~4 fold following treatment with 10^{-9}M E_2 as measured

5   by the nuclear binding assay (Figure 9, panel A). Similarly, the number of total cellular progesterone receptors increased ~2.5 fold, as measured by the DCC assay (Figure 9, panel B).

3.   Effect of E_2 on *c-fos* mRNA Levels in Subclone hFOB/ER9

10   E_2 treatment of hOB cells is known to result in a rapid increase in endogenous *c-fos* steady state mRNA level (S.A. Harris et al., J. Bone and Mineral Res., 7, 57 (1992)). To examine whether E_2 treatment of hFOB/ER cells has a similar effect on *c-fos* expression, northern analyses using the hFOB/ER9 subclone were performed. These data (Figure 10) showed that the

15   steady state level of endogenous *c-fos* mRNA increased ~3 fold within 30-60 minutes of E_2 (10^{-8}M) treatment of quiescent hFOB/ER9 cells cultured at 39.5°C. Interestingly, the basal (control) level of *c-fos* mRNA was ~4 fold higher in hFOB/ER cells maintained at 33.5°C than cells at 39.5°C. In addition no significant increase in the level of *c-fos* mRNA occurred in cells

20   cultured at 33.5°C.

D.   Deposit of Vector

          The ER plasmid pHEGO-HYG was deposited with the American Type Culture Collection (ATCC) on April 20, 1994, and assigned accession

25   number 79994 for the plasmid in *E. coli* HB 101 and 79995 for purified plasmid DNA.

          The complete disclosure of all patents, patent documents, and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding

30   only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for

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variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. An immortalized normal human fetal osteoblastic cell which expresses a temperature sensitive mutant of simian virus 40 large T protein antigen.

2. The immortalized normal human fetal osteoblastic cell of claim 1 wherein the cell is part of a homogeneous population.

3. The immortalized normal human fetal osteoblastic cell of claim 2 wherein the homogeneous population is a clonal population.

4. The immortalized normal human fetal osteoblastic cell of claim 1 which is nontumorigenic.

5. The immortalized normal human fetal osteoblastic cell of claim 1 wherein the T antigen is capable of being inactivated.

6. The immortalized normal human fetal osteoblastic cell of claim 5 wherein the T antigen is capable of being cycled between an inactive and an active state.

7. The immortalized normal human fetal osteoblastic cell of claim 1 which is capable of osteoblastic differentiation.

8. The immortalized normal human fetal osteoblastic cell of claim 1 which further expresses human estrogen receptor at a level greater than about 400 activated receptors per nucleus.
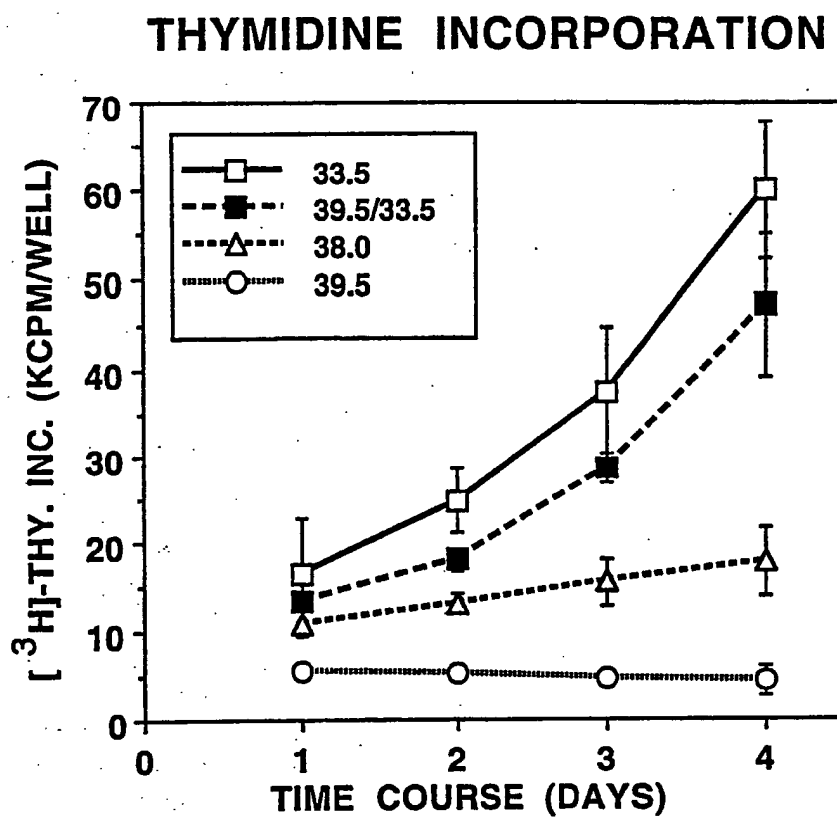
9. An immortalized normal human fetal osteoblastic cell having the identifying characteristics of ATCC CRL 11372.

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10. An immortalized normal human fetal osteoblastic cell prepared by a
     process comprising transfecting a human fetal bone cell with a gene
     coding for a temperature sensitive mutant of simian virus 40 large T
     antigen.

5

11. The immortalized normal human fetal osteoblastic cell of claim 10
     wherein the process further comprises transfecting the cells with a gene
     coding for a selectable marker.

10   12. The immortalized normal human fetal osteoblastic cell of claim 10
     wherein the process further comprises transfecting the cells with a gene
     coding for human estrogen receptor.

13. The immortalized normal human fetal osteoblastic cell of claim 12
15   wherein the gene coding for the human estrogen receptor is located on a
     replicable expression vector.

14.. The immortalized normal human fetal osteoblastic cell of claim 13
     wherein the replicable expression vector is a plasmid.

20

15. The immortalized normal fetal osteoblastic cell of claim 14 wherein the
     plasmid has the identifying characteristics of plasmid pHEGO-HYG.

16. The immortalized normal fetal osteoblastic cell of claim 14 wherein the
25   plasmid is pHEGO-HYG (ATCC No. 79995).

17. An immortalized normal human fetal osteoblastic cell that expresses
     human estrogen receptor at a level greater than about 400 activated
     receptors per nucleus.

30

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18. The immortalized normal human fetal osteoblastic cell of claim 17 wherein the gene coding for human estrogen receptor is contained in expression vector pHEGO-HYG.

5 19. An immortalized normal human fetal osteoblastic cell prepared by a process comprising transfecting a human fetal bone cell with expression vector pUCSVtsA58.

20. The immortalized normal human fetal osteoblastic cell of claim 19
10 wherein the process further comprises transfecting the human fetal bone cell with expression vector pSV2neo.
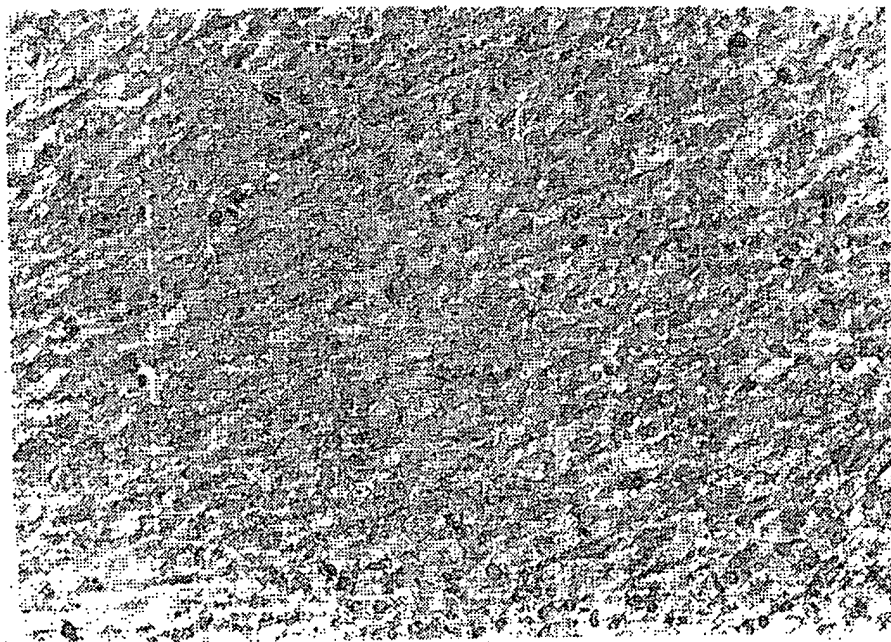
21. The immortalized normal human fetal osteoblastic cell of claim 19 wherein the process further comprises transfecting the human fetal bone
15 cell with expression vector pHEGO-HYG.

22. A method of preparing an immortalized normal human fetal osteoblastic cell comprising:
   (a) isolating a bone cell from human fetal tissue; and
20 (b) transfecting the isolated human fetal tissue cell with an expression vector comprising a gene coding for a temperature sensitive mutant of simian virus 40 large T antigen to yield said immortalized normal human fetal osteoblastic cell which expresses said antigen.
25

23. The method of claim 22 wherein the expression vector is a plasmid expression vector.

24. The method of claim 22 further comprising a step of transfecting the
30 isolated human fetal bone cell with a selectable marker gene.

40

25.   A method for testing a drug for effects on osteoblastic cell physiology
       comprising exposing a culture of immortalized normal human fetal
       osteoblastic cells which express a temperature sensitive mutant of simian
       virus 40 large T antigen to a drug and monitoring at least one change in
5   the physiology of said cultured cells.

26.   The method of claim 25 wherein the immortalized human fetal
       osteoblastic cells are cultured at a temperature no greater than about
       37°C.
10

27.   The method of claim 25 wherein the immortalized human fetal
       osteoblastic cells are cultured at a temperature greater than about 37°C.

28.   The method of claim 25 wherein the immortalized cells have the
15   identifying characteristics of ATCC CRL 11372.

29.   The method of claim 25 wherein the immortalized normal human fetal
       osteoblastic cells further express human estrogen receptor at a level
       greater than about 400 activated receptors per nucleus.
20

30.   The method of claim 27 wherein the monitoring step comprises
       monitoring secretion of growth factors and other cytokines, cell growth,
       expression of osteoblast associated genes, formation of mineralized
       nodules, mineralization of an extracellular matrix, or formation of bone.
25

31.   A method of treating bone loss comprising placing an effective bone-
       generating amount of immortalized human fetal osteoblastic cells which
       express a temperature sensitive mutant of simian virus 40 large T
       antigen into a deteriorated bone at the point of deterioration.
30

32.   The method of claim 31 wherein the deteriorated bone is a fractured
       bone and the point of deterioration is a point of fracture or break.

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33. The method of claim 31 wherein the wherein the immortalized cells have the identifying characteristics of ATCC CRL 11372.

34. The method of claim 31 wherein the immortalized human fetal
5   osteoblastic cells further express the human estrogen receptor at a level greater than about 400 activated receptors per nucleus.

THYMIDINE INCORPORATION

Fig. 1

Fig. 2B
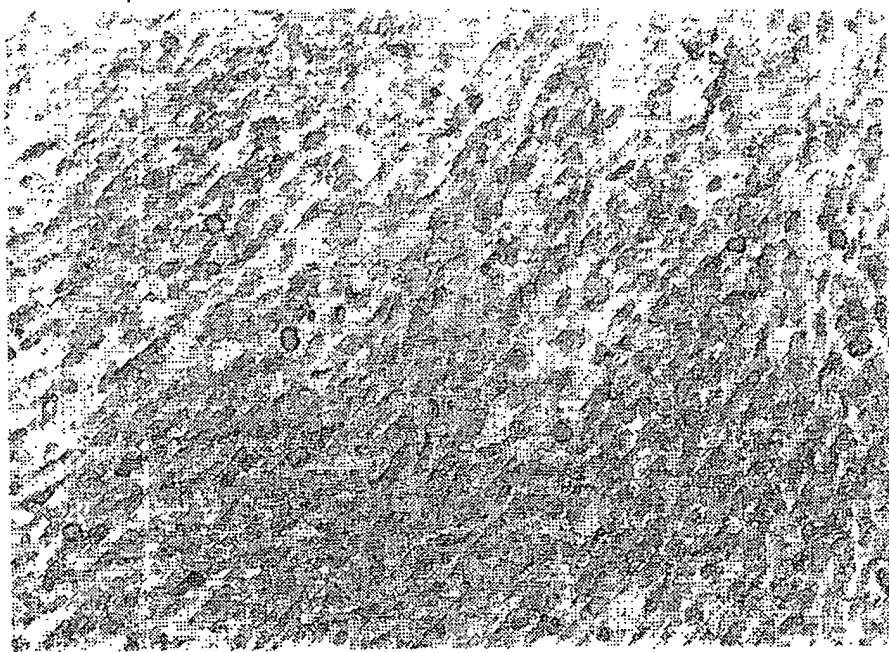
Fig. 2A

3/17

Fig. 2D
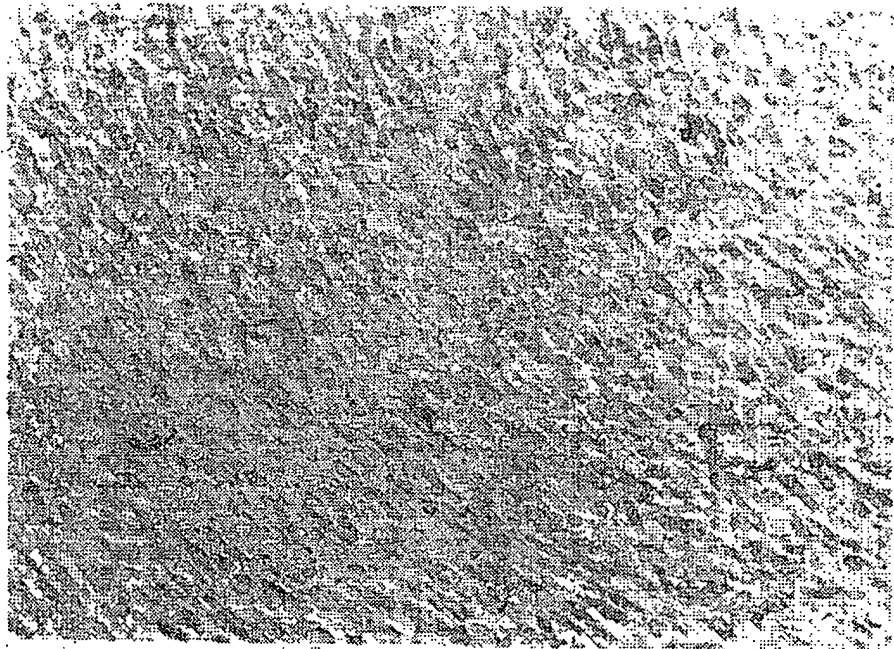
Fig. 2C
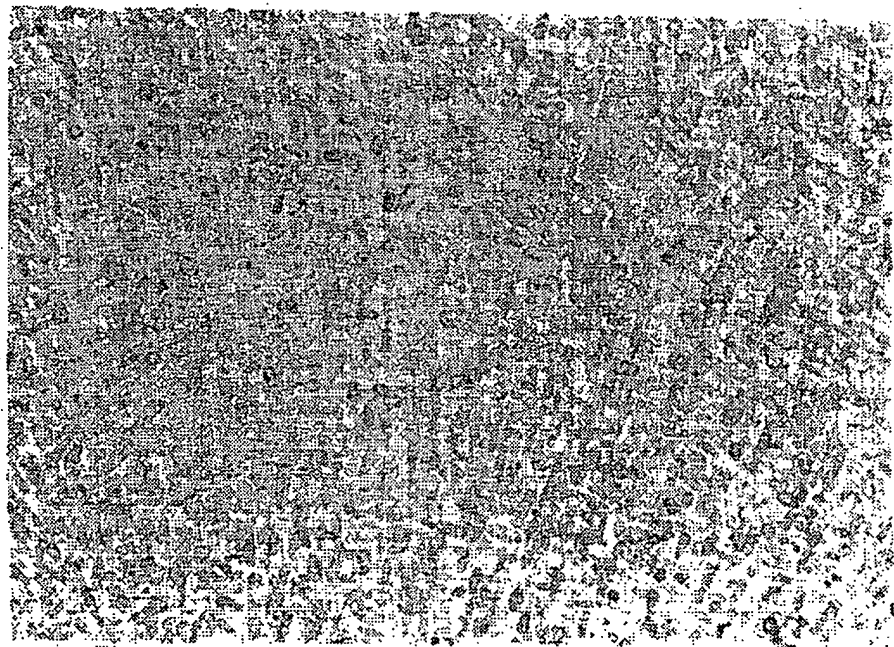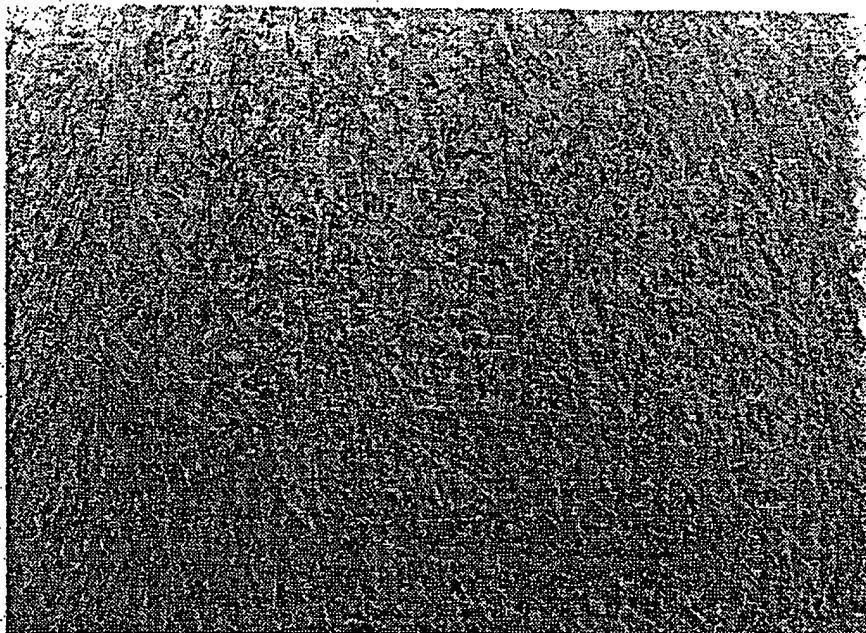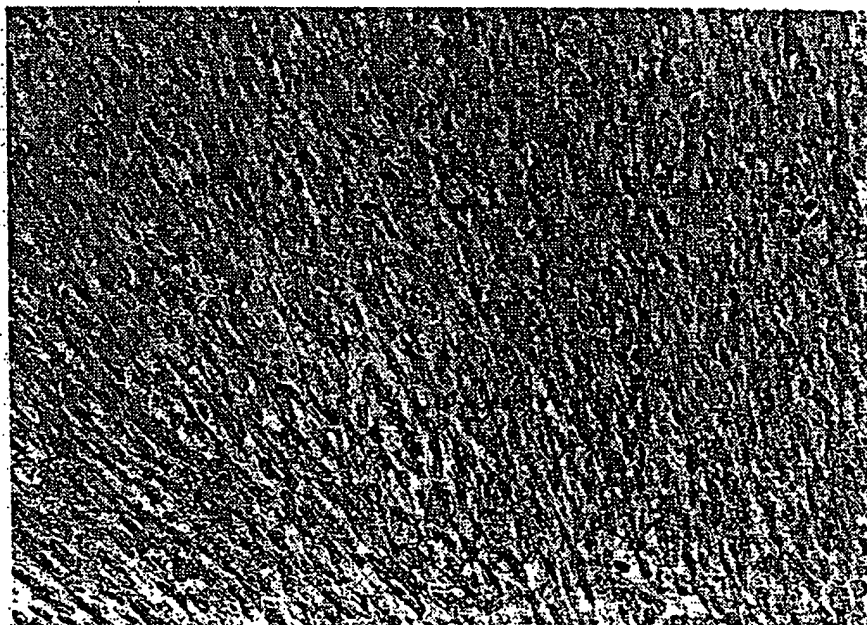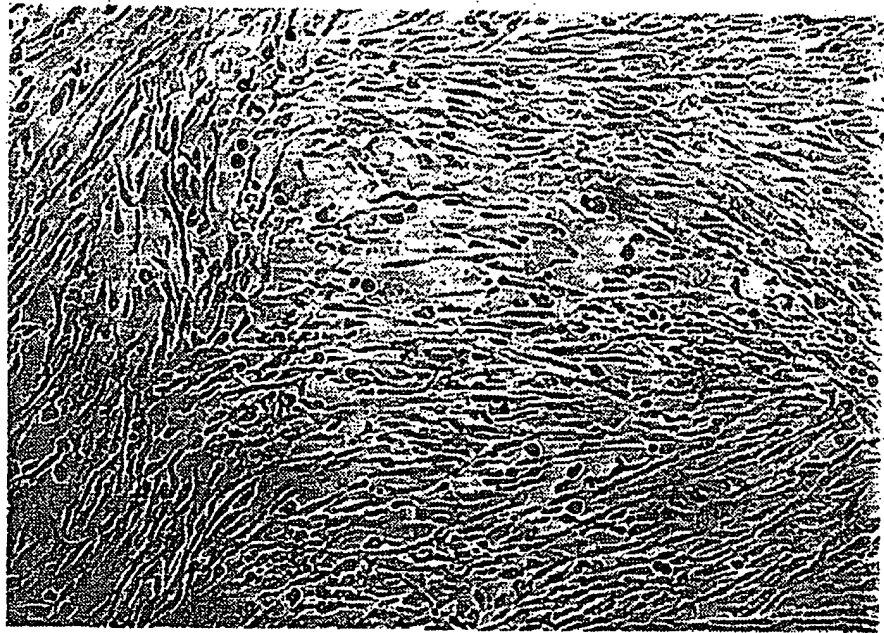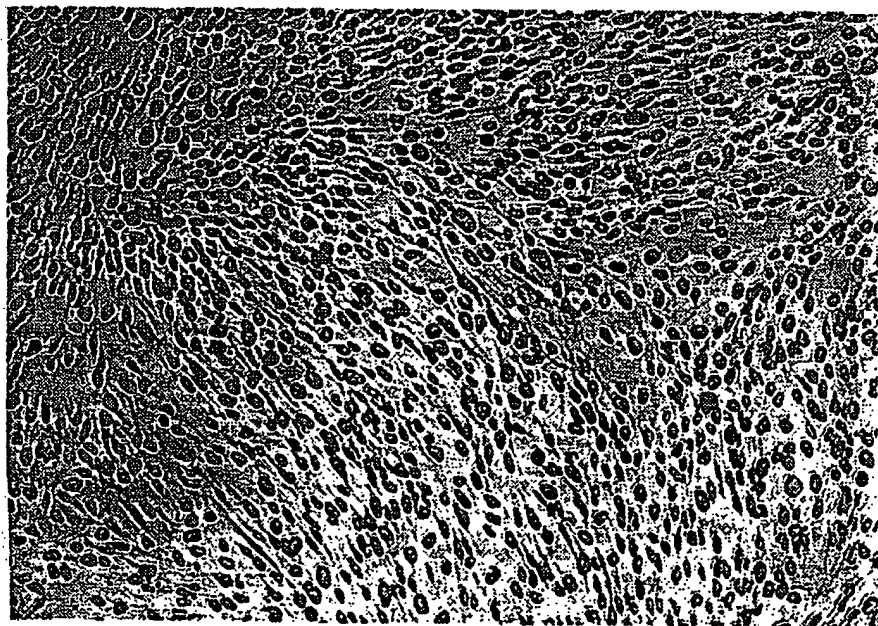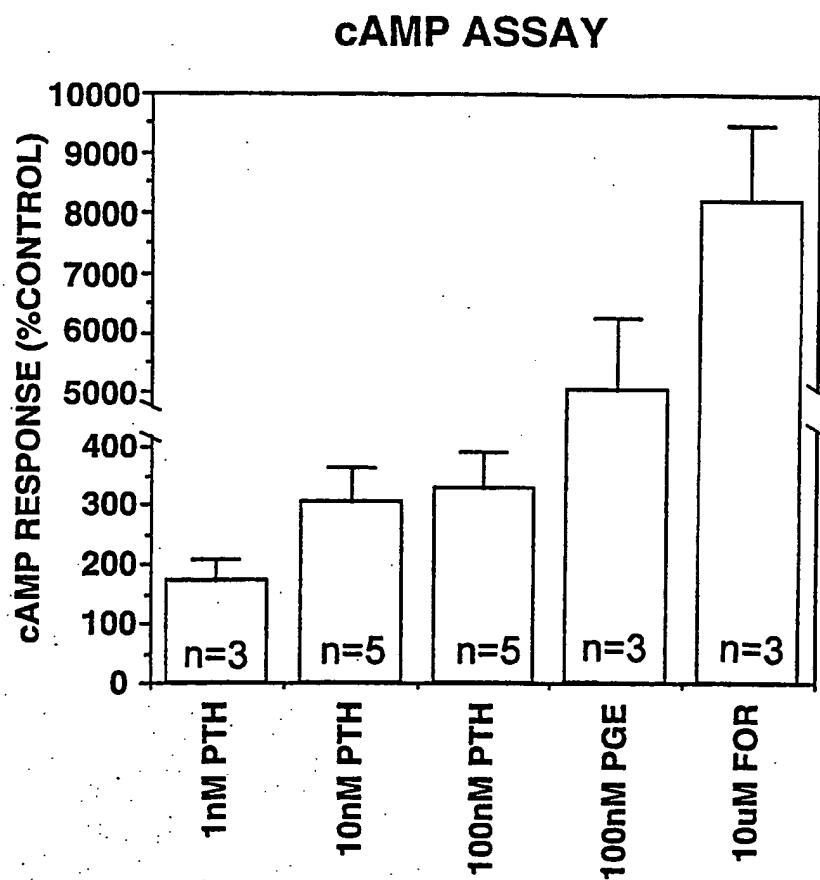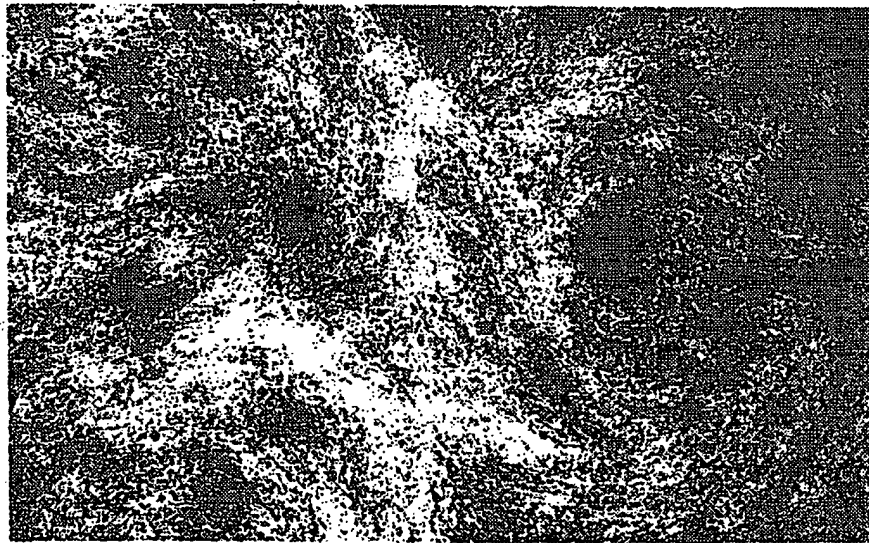
Fig. 2F

Fig. 2E

Fig. 2H

Fig. 2G

Fig. 3

Fig. 4B

Fig. 4A

Fig. 4D

Fig. 4C

Fig. 4E

Fig. 5B

Fig. 5A

Fig. 5D

Fig. 5C

ALKALINE PHOSPHATASE ASSAY
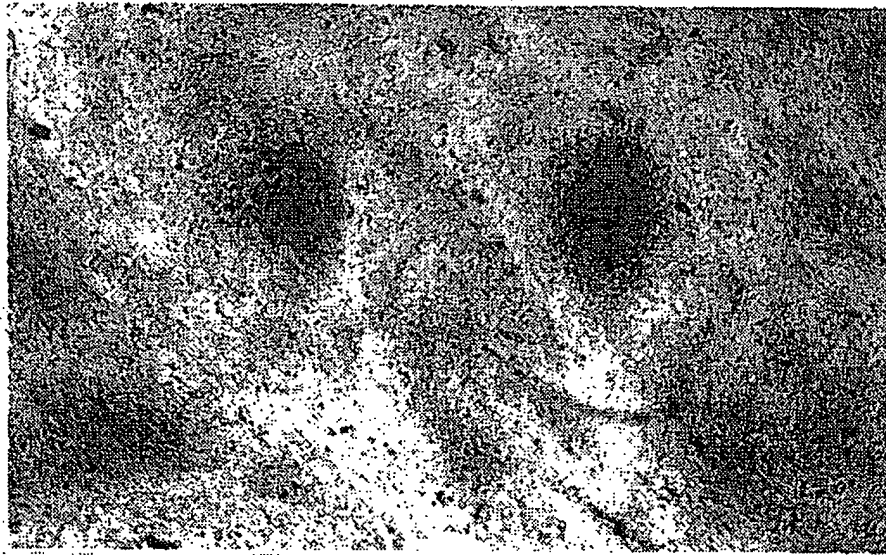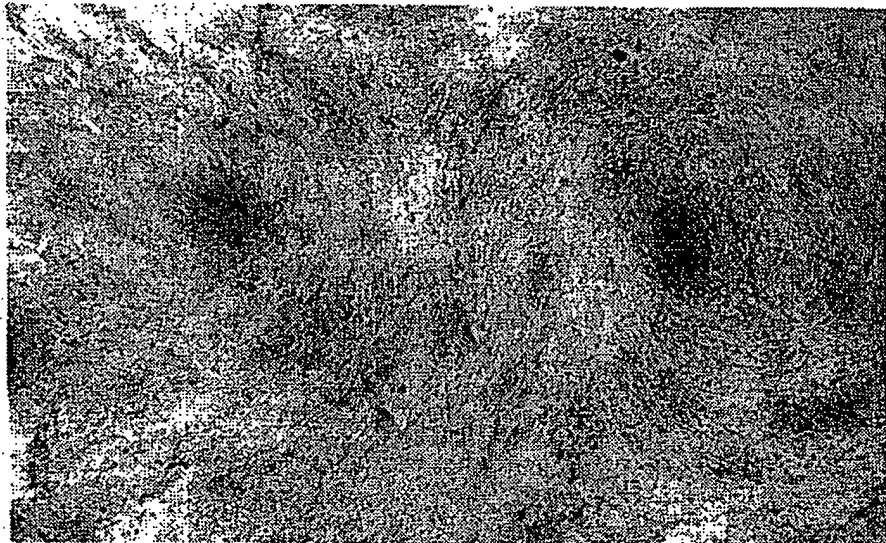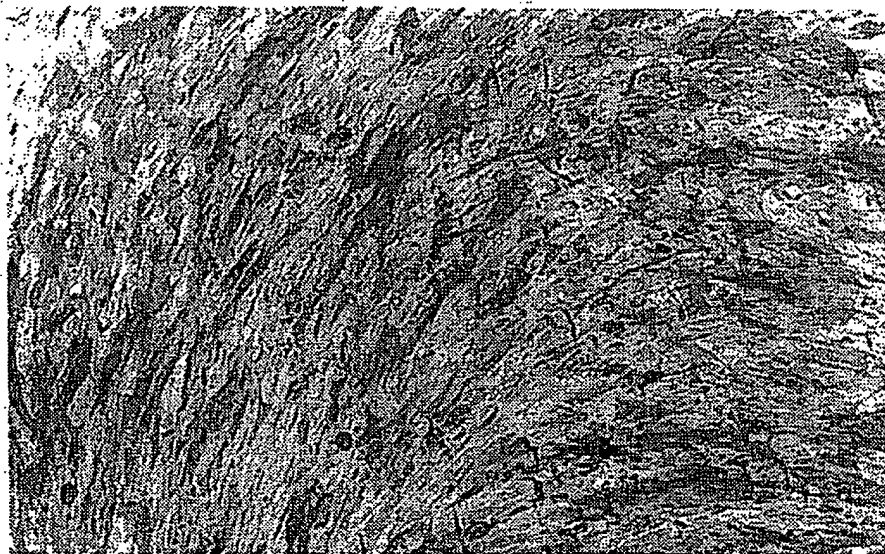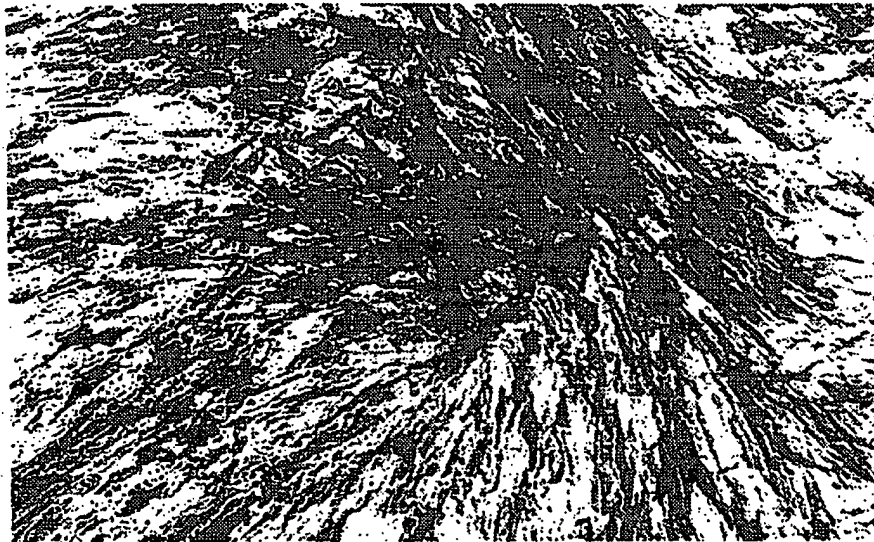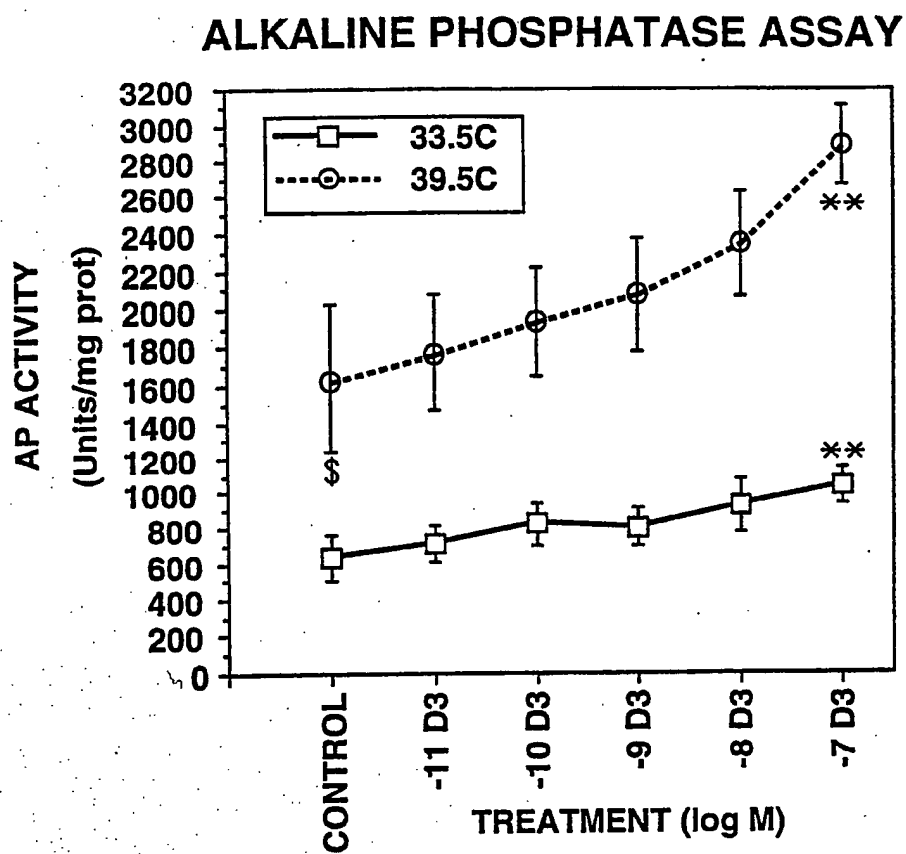
Fig. 5E

OSTEOCALCIN ASSAY

Fig. 6
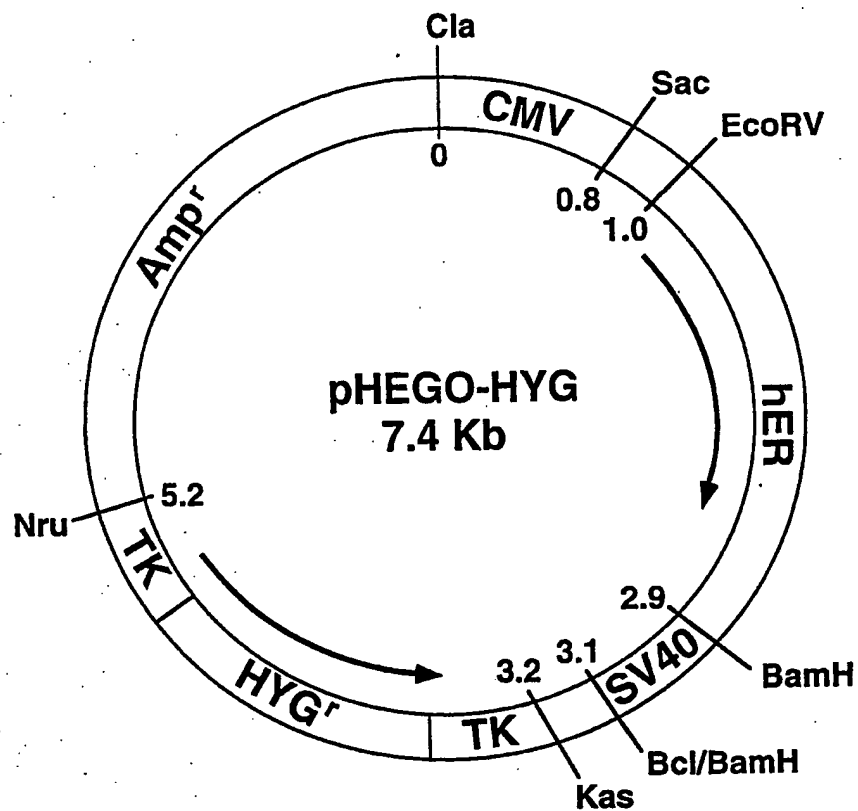
Fig. 7

hFOB/ER Subclones
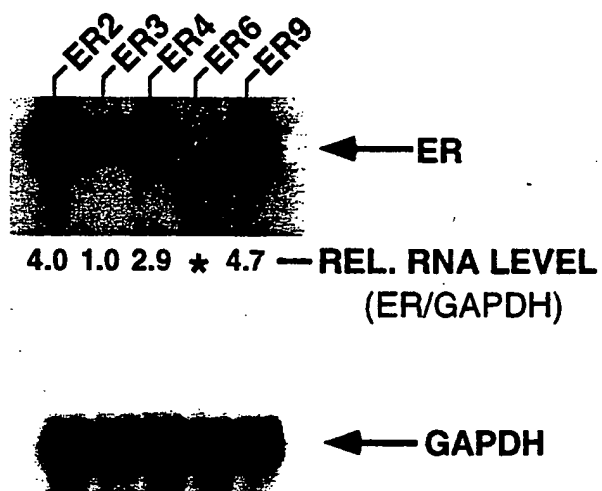
Fig. 8
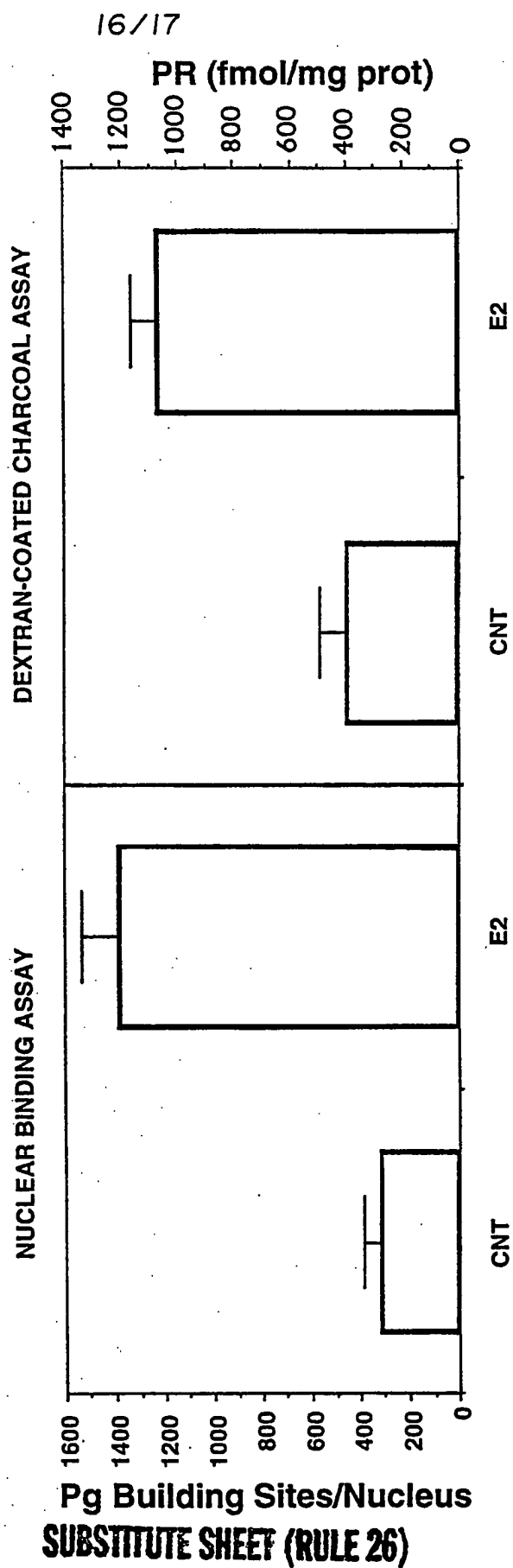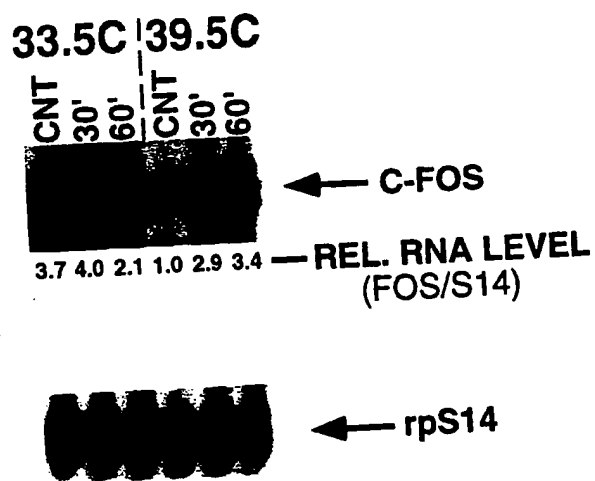
16/17

Fig. 9

Fig. 10

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N5/22   C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BONE AND MINERAL RESEARCH, vol.7, no.2, 1992, UNITED STATES pages 127 - 136 KEETING PE ET AL 'Development and characterization of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV40 large T antigen.' cited in the application	1,8,10, 12,17, 22,25,29
Y	JAPAN JOURNAL OF CANCER RESEARCH, vol.84, March 1993, JP pages 290 - 297 HIDEKI CHIBA ET AL. cited in the application see the whole document	1,8,10, 12,17, 22,25,29

-/--

| X | Further documents are listed in the continuation of box C. | X | Patent family members are listed in annex. |

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 October 1994	28. 11. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Gurdjian, D

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2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,89 09816 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 19 October 1989 see claim 7; example 2 ---	1,8,10, 12,17, 22,25,29
Y	JOURNAL OF STEROID BIOCHEMISTRY, vol.34, no.1-6, 1989, ENGLAND pages 483 - 490 WATTS CK ET AL 'Stable transfection of the oestrogen receptor gene into a human osteosarcoma cell line.'	8,12,17, 29
A	see the whole document ---	13
A	THE AMERICAN JOURNAL OF MEDICINE, vol.85, December 1988, US pages 847 - 850 BARZEL U.S. cited in the application see the whole document ---	8,12,17, 29
A	CHEMICAL ABSTRACTS, vol. 121, no. 13, 26 September 1994, Columbus, Ohio, US; abstract no. 154804, MARUSIC, ANA ET AL 'Production of leukemia inhibitory factor mRNA and protein by malignant and immortalized bone cells' see abstract & J. BONE MINER. RES. (MAY 1993), 8(5), 617-24 ---	1,10,22, 29
A	ENDOCRINOLOGY, vol.130, no.5, 1992, UNITED STATES pages 2617 - 2624 MIGLIACCIO S ET AL 'Estrogens modulate the responsiveness of osteoblast-like cells (ROS 17/2.8) stably transfected with estrogen receptor.' see the whole document -----	8,12,13, 17,18,29

2

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   31-34
   because they relate to subject matter not required to be searched by this Authority, namely:
   see PCT-Rule 39.1(1v)

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.: ..
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [·] As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WC-A-8909816	19-10-89	DE-T-	68907220	27-01-94
		EP-A,B	0428519	29-05-91
		JP-T-	3504799	24-10-91
		US-A-	5338839	16-08-94
		US-A-	5270191	14-12-93